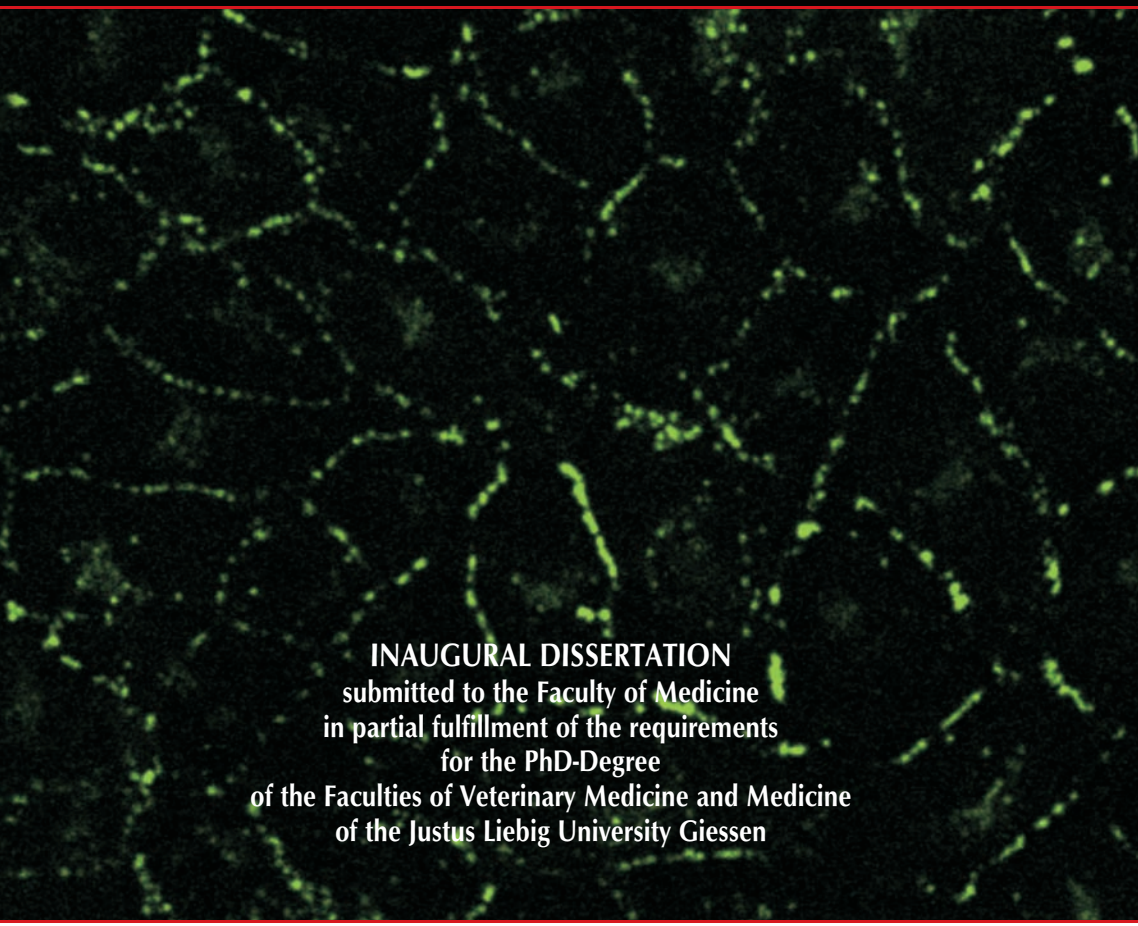
# Involvement of connexin 43 in ATP release from endothelial cells during reoxygenation. Role of PKA signaling pathway

KIRAN KHAWAJA



INAUGURAL DISSERTATION
submitted to the Faculty of Medicine
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of the Faculties of Veterinary Medicine and Medicine
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by

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of

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Giessen (2011)

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*Dedicated to:*

*My Family*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AAP10 | Antiarrhythmic peptide 10 |
| ABC | ATP binding cassette |
| adenosine | ADO |
| app. | Approximately |
| APS | Ammonium persulfate |
| ATP | Adenosine-5-triphosphate |
| BSA | Bovine serum albumin |
| $CaCl_2$ | Calcium chloride |
| cAMP | 3'-5'-cyclic adenosine monophosphate |
| Cx | Connexins |
| Cx37 | Connexin 37 |
| Cx40 | Connexin 40 |
| Cx43 | Connexin 43 |
| Da | Daltons |
| DMSO | Dimethyl sulfoxide |
| DTT | Dithiothreitol |
| EC | Endothelial cells |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylene diamine tetraacetic acid |
| EGTA | Ethylene glycol-bis (2-aminoethylether) N,N,N',N'-tetraacetic acid |
| Epac | Exchange protein directly activated by cAMP |
| Gap junctions | Gj |
| HBSS | Hanks' balanced salt solution |
| HEPES | 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid |

| | |
|---|---|
| HRP | Horseradish peroxidase |
| HUVEC | Human umbilical vein endothelial cells |
| Inxs | Innexins |
| I-R | Ischemia-Reperfusion |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| kDa | Kilo Dalton |
| LDH | Lactic acid dehydrogenase |
| MAPK | Mitogen-activated protein kinase |
| $MgCl_2$ | Magnesium chloride |
| min | Minutes |
| NaCl | Sodium chloride |
| $NaHCO_3$ | Sodium bicarbonate |
| $Na_2HPO_4$ | Sodium pyrophosphate |
| $NaH_2PO_4$ | Sodium dihydrogen phosphate |
| PAEC | Porcine aortic endothelial cells |
| Panxs | Pannexins |
| PBS | Phosphate-buffered saline |
| pH | Negative log of H+ concentration |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKI | Protein kinase A inhibitor |
| PMN | Polymorphonuclear leukocytes |
| ROS | Reactive oxygen species |
| Ser 368 | Serine 368 |
| SDS | Sodium dodecyl sulfate |
| TBS | Tris-buffered saline |

| | |
|---|---|
| TEMED | N, N, N', N',-tetramethylethylenediamine |
| Tris | Tris (hydroxymethyl) aminomethane |
| % vol/vol | Volume by volume percentage |
| % wt/vol | Weight by volume percentage |
| VRACs | Volume-regulated anion channels |

# 1 INTRODUCTION

## 1.1 Endothelial barrier function

Endothelial cells (EC) form a single cell layer of the inner surface of all blood vessels. This single cell layer, called endothelium, acts as a selective barrier for water, solutes, macromolecules and cells and controls the movement of all components between the tissue and streaming blood. Under physiological conditions endothelial cells are not only involved in barrier function but also in regulation of a variety of processes like angiogenesis, hemostasis, and blood flow (Stevens et al., 2001). Failure of endothelial barrier function may occur under pathophysiological conditions, like inflammation, sepsis or ischemia-reperfusion, which may cause local or systemic edema formation leading to acute life-threatening complications (Mehta and Malik, 2006; Michel and Curry, 1999). The mechanisms by which the endothelial barrier is maintained under physiological conditions as well as those processes leading to barrier failure are topics of many studies aiming to identify new strategies to protect the endothelial barrier.

Recently, it was shown that ATP, released from endothelial cells in response to reperfusion, can protect endothelial barrier against reperfusion-induced failure (Gündüz et al., 2006). The ATP release mechanism involved in this process, however, is unknown. The present study aimed to analyze this release mechanism in a model of cultured endothelial cells from porcine aorta.

## 1.2 Disturbance of endothelial barrier function

Endothelial cells are among the first targets of noxious processes induced during inflammation, ischemia-reperfusion, traumatic tissue injury or coagulation. Therefore, the failure of barrier function is one of the earliest signs of cell injury causing extravasation of plasma components and cells of the bloodstream and finally leading to organ dysfunction. Disturbance of endothelial cells which takes place in response to reperfusion of the ischemic heart causes obstruction of microvessels and prevents restoration of blood flow of the previously ischemic myocardium. This so called “no-

reflow phenomenon" (Kloner et al., 1974), resulting from endothelial ischemia-reperfusion injury, is a critical determinant for the myocardial infarction. During reperfusion, barrier failure due to endothelial cell injury may be induced by many exogenous factors involving leucocyte or platelet-interaction with endothelial cells, production of reactive oxygen species (ROS) released from activated leucocytes or myocytes or the complement system (Eltzschig and Collard, 2004). However, barrier failure may also be triggered by endogenous endothelial mechanisms. As shown in cultured endothelial cells as well as in isolated saline-perfused hearts (Gündüz et al., 2006), the principle endothelial effector mechanisms involved in the acute increase in permeability during reperfusion are: loss of cell-cell or cell-matrix adhesion, disassembly of the endothelial cytoskeleton and activation of the contractile machinery. These mechanisms cause retraction of endothelial cells and formation of intercellular gaps (Gündüz et al., 2006; Schäfer et al., 2003). Ischemia-reperfusion may also cause shedding of the endothelial glycocalyx (Rehm et al., 2007), which represents an important determinant of physiological endothelial barrier (Becker et al., 2010).

## 1.3 Release of ATP from cardiovascular cells

Forrest and Williams (1977) are among the first who reported that ATP is released from isolated cardiomyocytes under normoxic conditions and that the release was enhanced when the cells were exposed to hypoxia. Today, it is well established that all cells of the cardiovascular system release ATP into the interstitial space or, in case of endothelial cells, also directly into the vascular lumen (Burnstock, 2002; Clarke, 2009; Faigle et al., 2008; Gomes et al., 2005). This release is enhanced due to shear stress (Bodin and Burnstock, 2001; Yegutkin et al., 2000), oxidative or osmotic stress (Taylor et al, 1998; Schwiebert et al, 2002), and during blood coagulation (Gordon et al., 1986), inflammation (Bodin and Burnstock, 1998), or ischemia-reperfusion (Bergfeld and Forrester, 1992; Clemens and Forrester, 1981). After being released, ATP binds to cell surface receptors like $P_2Y$ and $P_2X$ and may regulate the vascular tone (Burnstock, 2006). $P_2Y$ receptors may act via $Ca^{+2}$-dependent eNOS activation, which causes nitric oxide production, while $P_2X$ receptors may act as ATP sensors (Khakh and North,

2006). Sometimes, ATP undergoes degradation leading to the formation of adenosine (ADO), which causes arterial vasodilation via $A_2$-adenosine receptors (Burnstock, 2002).

Extracellular ADO and ATP have been shown to be protective during hypoxia or ischemia (Baxter, 2002; Linden, 2001). In addition, ATP has also been found to be released in response to a hypoxic or an ischemic stimulus, and the increase in extracellular ATP can help in stabilization of endothelial barrier function via purinergic signaling (Noll et al., 2000; Gündüz et al., 2006; Eltzschig et al., 2006). ATP reduces the permeability of microvascular coronary endothelial cells. It not only stabilizes the barrier but also reduces the thrombin-induced loss of barrier function (Gündüz et al., 2003; Noll et al., 2000; Klingenberg et al., 2004). If ATP degradation is prevented, it reduces myocardial edema formation in response of reperfusion (Gündüz et al., 2006).

Although it is well established that vascular cells release ATP, the molecular and signaling mechanisms involved are still not known. Various possible mechanisms may come into consideration including volume-regulated anion channels (VRACs), vesicular exocytosis, ATP binding cassette (ABC) transporters, plasmalemmal voltage-dependent anion channels, $P_2X_7$-receptor channels, as well as connexin or pannexin hemichannels (Goodenough and Paul, 2003; Fiagle et al., 2008; Leybaert et al., 2003; Stout et al., 2002; D'hondt et al., 2009).

## 1.4 Connexin

### 1.4.1 Connexins and connexons

Endothelial cells form a variety of cell-cell contacts such as tight junctions (Rubin, 1992), adhesion junctions (Rubin, 1992), and gap junctions (Goodenough and Paul, 2003; Ebihara, 2003). Gap junctions (Gj) form connections between the cytoplasm of two adjacent cells. Each cell contributes half of the intercellular channel, also known as hemichannel or connexon. Each hemichannel is composed of six protein subunits termed connexins (Cx). One hemichannel in the plasma membrane docks to another hemichannel of the adjacent cell and forms a complete gap junctional channel. Ions and small molecules with a mass of up to 1 kDa can diffuse through gap junctional channels allowing electrical and metabolic coupling of cells (Goodenough and Paul, 2003).

A connexon may be homomeric (contains single type of connexins) or heteromeric (contains multiple connexins). In addition, neighbouring cells could contribute homomeric or heteromeric connexons also called homotypic or heterotypic intercellular channels. Due to this fact, large varieties of Cx channels are available with different properties of permeability, selectivity, conductance, and gating (Bukauskas et al., 2002; Elfgang et al., 1995; Goldberg et al., 2004; Grikscheit et al., 2008; Rackauskas et al., 2007).

There are 20 members in Cx gene family in the mouse and 21 members in the human genome. The structure of connexins is highly conserved among the Cx family members. It consists of four transmembrane domains. One intracellular loop and two extracellular loops with six conserved cysteine residues are involved in Gj formation (Söhl and Willecke, 2004). The C-terminal end is in the cytosol and varies in length, causing the different molecular masses of Cx. This C-terminal end has been shown to be phosphorylated by many kinases regulating the open propability of Cx, while the N-terminal end, also located in the cytosplasm, is not (Lampe and Lau, 2000). The C-terminal domain is usually involved in controlling different functions by providing the site of protein-protein interactions (Söhl and Willecke, 2004).

Generally, the Gj formation starts either within the endoplasmic reticulum or in the trans-golgi network, where the formation of newly synthesized Cx into connexons takes place. The exception is Cx26, which seems to bypass the trans-golgi network (Evans et al., 2006). After being synthesized inside the endoplasmic reticulum, the connexons are transported via vesicles towards the plasma membrane, where they get inserted. However, to form the Gj, the connexons move towards the Gj plaque and dock with Gj plaques of adjacent cells to form complete Gj channel (Ebihara, 2003).

Cx are short-lived with half-life of about 1-3 h, as they are regularly degraded. The degradation process could be via one of the following routes: (1) via proteosomes including endoplasmic reticulum degradation, (2) by lysosomes prior to reaching the plasma membrane or (3) or, if already integrated into Gj, Cx plaques are degraded by lysosomes after endocytotic internalization. Degradation process involves ubiquitination and phosphorylation (Berthoud et al., 2004).

Connexons have also been detected at the cell surfaces, forming so called Cx hemichannels that are not part of intercellular Gj. Generally these hemichannels remain in a closed state on the surface of most cells. Cx activation might be a common response to metabolic inhibition (Goodenough and Paul, 2009). However, the exact signaling mechanism involved in connexin hemichannel opening is still unclear.

### 1.4.2 Role of connexins in the cardiovascular system

The vascular wall consists of layered smooth muscle cells and the endothelium both of which are connected by Gj (Little et al., 1995). Depending upon the cell type and the compartment, four types of Cx have been detected in the vascular wall, i.e., Cx37, Cx40, Cx43, and Cx45 (Haefliger et al., 2004). In endothelial cells, major forms of Cx are Cx37, Cx40 and Cx43. The distribution suggests that Cx37 and Cx43 are involved in more diverse processes than Cx40 (Sáez et al., 2003).

Studies have been undertaken to elucidate the function of cardiovascular Cx. The importance of Cx43 has been explained with the experiments performed on Cx43 null mice. These animals survive until birth. At the time of birth, they have a beating heart but they die shortly after birth due to obstruction of the right ventricle (Sáez et al., 2003). Heterozygous Cx43 mice (Cx43 +/-), however, have slow epicardial conduction in the ventricle (Guerrero et al., 1997; Thomas et al., 1998).

### 1.4.3 Regulation of connexins

Cx are regulated by interacting with other cellular proteins. Usually this regulation is mediated by phosphorylation and has been shown by incorporation of $^{32}P$. 12 of the 21 serines (Lampe and Lau, 2004) and 2 of the tyrosines of Cx43 are phosphorylated by several different kinases (Solan and Lampe, 2009; Lampe and Lau, 2004).

With the exception of Cx36 and Cx56, which can be also phosphorylated in the cytoplasmic loop region, Cx are mainly phosphorylated in the C-terminal domain (Solan and Lampe, 2009). There is no evidence of phosphorylation of the N-terminal region (Lampe and Lau, 2004). As Cx26 is the shortest Cx among all, this protein does not show phosphorylation at all (Traub et al., 1989).

The most commonly expressed Cx is Cx43, as its presence has been detected in 34 tissues and 46 cell types (Laird, 2006). Cx43 is most probably the best analyzed Cx in regard to phosphorylation and function. No serine residue has been detected in the cytoplasmic loop of Cx43. Cx43 can be phosphorylated by the activation of several different kinases such as protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and $pp60^{src}$ kinase (Src) (Solan and Lampe, 2009).

Previous studies have shown that PKA causes an increase of Gj synthesis as well as electrical coupling of cardiac cells (Imanaga et al., 2004). Maneuvers inducing an upregulation of cAMP also cause an increase in Cx43 phosphorylation and intercellular communication (TenBroek et al., 2001). These effects could be suppressed by PKA inhibitors (Matsumura et al., 2006). Such evidence suggests that the effect of cAMP on the Gj function is mediated by increase in phosphorylation of Cx43 in a PKA dependent manner (Imanaga et al., 2004). Furthermore, it was suggested that PKA-mediated phosphorylation of Cx43 enhances open probability of Gj, intercellular coupling (Imanaga et al., 2004) and either a delay in the degradation or acceleration of the synthesis of connexin protein (Matsumura et al., 2006).

In contrast, it has been shown that activation of PKC or MAPK results in a phosphorylation of the Cx43 and reduction of open probability of Cx43 hemichannels (Warn-Cramer et al., 1998; Kim et al., 1999). Increase in Cx43 phosphorylation is associated with an increase in reduced Gj communication, and reduced electrical coupling (Moreno, 2005). On the other hand, Cx43 hemichannel permeability increases with phosphatase treatment (Kim et al., 1999).

## 1.5 Connexins and ATP release

Cx as hemichannels are known to be involved in the paracrine communication as they facilitate trafficking of substances across cell membranes into the interstitial space. One of them also includes the release of ATP (Clarke et al., 2009).

As mentioned earlier, ATP is released from many different cell types. For some cell types, this ATP release can be blocked by inhibitors of Cx, ABC-protein family members or nucleoside transporters (Wang et al., 2005). Regardingly, involvement of

Cx43 on ATP release was shown in neuronal astrocytes (Stout et al., 2002), PMN (polymorpho nuclear cells) (Eltzschig et al., 2008), cardiomyocytes as well as bovine corneal and human microvascular endothelial cells (Clarke et al., 2009; Faigle et al., 2008; Gomes et al., 2005) during ischemic conditions and shear stress.

## 1.6 Connexin channel openers

During myocardial infarction, disruption of Gj as well as reduction in Cx43 expression has been identified as critical determinants in genesis of arrhythmia. Therefore, the availability of agents like the hexapeptide AAP10, a so called antiarrhythmic peptide, which enhances the formation of gap junctions and intercellular coupling, represents a promising therapeutic option to prevent arrhythmia (Müller et al., 1997). The concept to enhance cell coupling by a pharmacological agent was further supported by ZP123, which is a much more stable chemical analogue of the AAP10 (Salameh and Dhein, 2005). ZP123 has similar effects as AAP10. It increases the intercellular communication, promotes electrical coupling, and attenuates gap junctional closure (Clarke et al., 2006). Although AAP10 is less stable than ZP123, it can be used to study the effect of connexin-mediated cell-cell communication. AAP10 enhances the metabolic coupling and the expression level of Cx43 via a PKC-dependent pathway (Easton et al., 2009).

Alendronate, a 250 Da synthetic molecule, belongs to a group of bisphosphonates. It is used for the treatment of several bone diseases and has anti-apoptotic effects on osteocytes and osteoblasts (Plotkin et al. 1999). Further studies revealed that alendronate may act as an opener of Cx43 hemichannels, which is assumed to induce a signaling pathway of cell survival, involving activation of Src and ERK kinase in response to an interaction with the C-terminal domain of Cx43 (Plotkin et al. 2002). The exact mechanism by which alendronate causes the opening of Cx43 as well as the activation of the survival cascade is still unknown.

## 1.7 Pannexins

Previously, it was thought that Gjs are formed only from Cxs in vertebrates. However, this conception was changed when another family of Gj proteins, called innexins (Inxs), was identified in invertebrates (Phelan, 2005). Orthologous of Inxs discovered in vertebrates are named as pannexins (Panxs) (Panchin et al., 2000). Although Cxs and Inxs/Panxs are formed independent of each other and show little sequence homology, they show many common features such as the ability to form Gj and facilitate intercellular communication. Hemichannels for Cx, Inx, or Panx are called connexons, innexons, or pannexons, respectively (D'hondt et al., 2009).

As mentioned earlier, Cxs have a short half-life of 3 hours depending on the cell type (Beardslee et al., 1998). On the other hand, Panxs have a half-life of more than 6 h, indicating that Panxs synthesis and expression might be regulated differently (Penuela et al., 2007). Like Cxs, Panxs can also be activated by different stimuli like shear stress, ATP or other agonists (D'hondt et al., 2009).

Cx channels are known to show high open probability when extracellular $Ca^{+2}$ levels are reduced (Thimm et al., 2005). In contrast, Panxs are unaffected by extracellular $Ca^{+2}$ levels (Bruzzone et al., 2005). On the other hand, open probability is enhanced when intracellular $Ca^{+2}$ level is increased (Locovei et al., 2006). It has been observed that Panxs channels open in response to agonist-induced increase in cytosolic $Ca^{+2}$ concentration (Locovei et al., 2006). Recently, it has been shown that Panx1 interacts with $P_2X_7$ receptor and may form a $P_2X_7$-Panxz1 complex (Kim and Kang, 2011). Release of ATP via Panxs has recently been observed in erythrocytes in response to lower oxygen tension (Sridharan et al., 2010) causing the activation of cAMP-dependent PKA pathway (Adderley.et al., 2010). However, the detailed mechanism of this ATP release is still unclear (Sridharan et al., 2010).

## 1.8 Aims and objectives of the study

In the present study, the hypothesis was tested whether vascular endothelial cells do release endogenous ATP during reoxygenation and keep barrier failure at bay. Previous studies in endothelial cells have demonstrated that ATP, when exogeneously applied during reperfusion, protects the endothelial barrier against reperfusion injury. The study was performed using an established model of cultured monolayers of porcine aortic endothelial cells (PAEC). The following questions were addressed.

- What is the mechanism involved in reoxygenation-induced ATP release in endothelial cells?
- Is the reoxygenation-induced ATP release Cx43 dependent?
- What role are Panxs, Cx37 and Cx40 playing in this ATP release?
- What is the signaling mechanism underlying the reoxygenation-induced ATP release?
- What is the role of Cx43 phosphorylation and how is this mechanism modulated by PKA?
- Do Cx channel openers enhance the protective effect of ATP release from endothelial cells?

# 2 METHODS

## 2.1 Preparation of endothelial cells

*Isolation and cultivation of endothelial cells from porcine aortas*

Porcine aortic endothelial cells (PAEC) were freshly isolated from porcine aortas of approx. 20 cm of length isolated at the slaughter house and transported in 0.9% (wt/vol) NaCl solution. Afterwards the aortas were cleaned from the outside by cutting off fat, loose tissue, and lymphatic nodes. After cleaning, the aorta was opened cutting along the intercoastal arteries, fixed on an aluminum foil covered styropor board and rinsed with 0.9% (wt/vol) NaCl solution. Subsequently the upper layer of endothelial cells was scraped with a scalpel and transferred into 40 ml of pre-warmed M199 medium. Harvested cells were pelleted by centrifugation for 10 min at 260 x g at room temperature, the medium was removed and the cells were resuspended in 40 ml fresh cell culture medium. Subsequently the cell suspension was spread onto primary culture dishes (approx. 3-4 aortas / dish) containing 10 ml of cell culture medium with 5% (vol/vol) penicillin/streptomycin. After 3 hours of incubation at 37 °C and 5% $CO_2$ cells were extensively washed with HEPES/Tyrode's buffer (HBS) to remove non-adherent cells and cell debris. Afterwards adherent endothelial cells were incubated in 15-20 ml of cell culture medium containing 5% (vol/vol) penicillin/streptomycin at 37 °C and 5% $CO_2$. After 24 hours the cell culture medium was changed to medium containing 2% (vol/vol) penicillin/streptomycin and incubation was continued at 37 °C and 5% $CO_2$.

**M199 / $CO_2$. Medium: (pH 7.4)**

| | |
|---|---|
| Medium 199 / Earl's Salts | 9.62 g/l |
| HEPES | 15 mM |
| $NaHCO_3$ | 24 mM |
| Carbogen | Perfused approx. 1 hour. |

**Cell culture medium:**

| | |
|---|---|
| M199 / $CO_{2-}$ medium | x ml |
| NCS (vol/vol) | 20% |
| Penicillin/streptomycin (100 lU/ml, 100 µg/ml) | 2% (vol/vol) |

**HEPES/Tyrode's buffer (HBS): (pH 7.4)**

| | |
|---|---|
| NaCl | 125 mM |
| KCL | 2.6 mM |
| $KH_2PO_4$ | 1.2 mM |
| $MgSO_4$ | 1.2 mM |
| HEPES | 25 mM |

## 2.2 Subcultivation of endothelial cells

Confluent monolayers of endothelial cell cultures were trypsinized 7 to 10 days after isolation. Cells were washed with HBSS ($KH_2PO_4$ 0.44 mM, KCL 5.37 mM, $Na_2HPO_4$ 0.34 mM, NaCl 136.89 mM, and D-Glucose 5.55 mM) and subsequently incubated with 3 ml of trypsin/EDTA solution (composition in mM: 137 NaCl, 2.7 KCL, 1.5 $KH_2PO_4$, 8.0 $Na_2HPO_4$, pH 7.4, 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA). Detached cells were collected into cell culture medium and cells were seeded at a density of $2.2x10^4$ cells/$cm^2$ on 35 mm dishes according to the experiment being performed. For immunostaining and confocal microscopy cells were seeded on 2.5 cm glass coverslips.

## 2.3 Application of pharmacological compounds

Stock solutions were prepared immediately before use in basal medium or DMSO. Appropriate volumes of these solutions were added to the cells yielding final solvent concentrations ≤ 0.1% (vol/vol). The same final concentration of DMSO was also included in all respective control experiments.

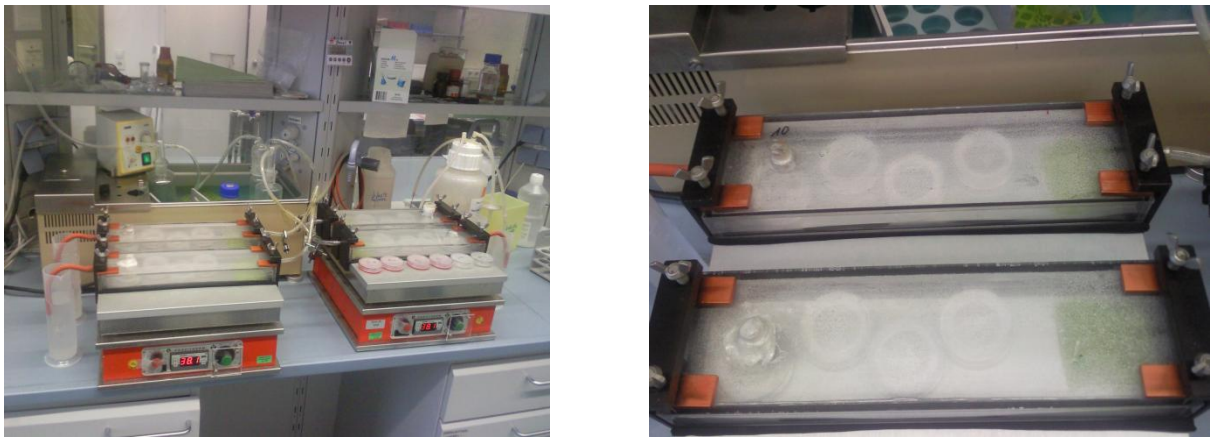
## 2.4 Experimental protocol for hypoxia

Sub-confluent monolayers of endothelial cells (80-90% confluent) were equilibrated on heating plates at 37 $^{o}$C for 40 min after changing the medium with HEPES buffer (NaCl 125 mM; KCl 2.6 mM; $MgCl_2$ 1.2 mM; $KH_2PO_4$ 1.2 mM; HEPES 25 mM; pH 7.4; 1.3 mM $CaCl_2$ was added just before use). Afterwards 40 min of hypoxia was produced in an air tight incubation chamber by exposing cells to a continuous stream of humidified $N_2$ gas ($Po_2$ approximately 10 mmHg), while the normoxic controls were exposed to humidified air ($Po_2$ = 140 mmHg). Afterwards cells were reoxygenated for 15 min by opening the airtight incubation chamber.

**Hypoxia medium**

| | |
|---|---|
| HEPES buffer | x ml |
| $CaCl_2$ | 1.3 mM |

**A** **B**



**Photograh 1: (A)** Photographs showing the air tight hypoxic incubation chambers through which a continuous stream of $N_2$ gas is passed. The chambers on the right side are exposed to humidified air as a normoxic control. **(B).** Close view of chambers showing the cell culture dishes exposed to $N_2$ gas.

## 2.5 Transfection of endothelial cells

In order to reduce endogenous Cx43 content, endothelial cells were transfected with Cx43-specific siRNA duplex and transfection reagent jetSI-ENDO 24 h prior to experiments according to manufacturer's instruction. Specific siRNA against porcine Cx43 was designed and produced by Eurogenetec (Köln, Germany) according to accession no: AY382593. The three different antisense sequences tested and used are as follows:

Cx43.1: 5'-AGAGAGGAAACAGTCCACC-3'.
Cx43.2: 5'-ATGAAGATGATGAAGATGG-3'.
Cx43.3: 5'-AACAGACACAAATATGATC-3'.

Non-specific control siRNA duplex from two different companies was used as a negative control. Most effective Cx43 silencing was achieved with concentrations of 100nM of duplex siRNA for 24 h.

## 2.6 Detection of ATP release

The amount of the ATP released into the cell culture medium of endothelial monolayers was determined with the luciferin-luciferase assay as described by Gündüz et al. (2006) with minor modifications. Cells in 35 mm dishes were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) or control normoxia ($Po_2$ = 140 mmHg) in gas tight chambers, respectively. Medium of endothelial monolayer was collected very carefully without touching the cells at the end of hypoxia and at different time points of reoxygenation. Three samples of 80 µl each were mixed with 80 µl of ATP luciferin-luciferase assay reagent in an eppendorf tube, vortexed and then immediately placed into a TD 20/20 Luminometer (Turner Designs) and measured for 10 s after a delay of 1 s. All assays were performed at room temperature. As control all pharmacological substances were tested with ATP standard performed in each individual experiment.

## 2.7 Detection of LDH release

The activity of cytoplasmic marker enzyme lactate dehydrogenase (LDH) was assayed by a commercial detection kit (CytoTox-One, Promega, Mannheim, Germany). LDH was

assayed in the supernatant of endothelial monolayers during reperfusion according to manufacturer's instructions. In short, confluent PAEC monolayers were exposed to hypoxia followed by reoxygenation, subsequently 100 µl of the substrate mixture from the kit were added to 100 µl of the collected protein-free supernatant. After an incubation of 30 min the absorbance was measured at 490 nm. In positive control samples, PAECs were treated with 9 % Triton X-100 to access the maximal release of LDH.

## 2.8 Protein analysis

### 2.8.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

| | |
|---|---|
| **Resolving gel buffer:** Tris/HCl; pH 8.8 | 120 mM |
| **Stacking gel buffer:** Tris/HCl; pH 6.8 | 120 mM |

### 2.8.2 Sample preparation

Endothelial cells were lysed in 200 µl SDS sample buffer (250 mM Tris/HCl; pH 6.8, 20 % (vol/vol) glycerol, 4 % (wt/vol) SDS, 1 % (vol/vol) mercaptoethanol, 0.001 % (wt/vol) bromphenol blue, and 10 mM DTT (added freshly before use). Subsequently, 50 IU/ml benzonase in 2 mM $MgCl_2$ was added and the lysate was collected in a 1.5 ml Eppendorf tube. Samples were denatured for 10 minutes at 65 °C and used immediately or stored at –20 °C.

**10 x Gel running buffer**

| | |
|---|---|
| Tris | 250 mM |
| Glycine | 2.0 M |
| SDS (wt/vol) | 10 % |

**Stacking gel 6%**

| | |
|---|---|
| Acrylamide 40% (wt/vol) | 3.8 ml |
| Bisacrylamide 2% (wt/vol) | 2 ml |
| Stacking buffer pH 6.8 | 6 ml |
| Demineralized water | 17.5 ml |

| | |
|---|---|
| SDS 10% (wt/vol) | 250 µl |
| APS 10% (wt/vol) | 200 µl |
| TEMED | 20 µl |

**Resolving gel 12.5%**

| | |
|---|---|
| Acrylamide 40% (wt/vol) | 12.7 ml |
| Bisacrylamise 2% (wt/vol) | 7 ml |
| Resolving buffer pH 6.8 | 9.5 ml |
| Demineralized water | 9.8 ml |
| SDS 10% (wt/vol) | 0.4 ml |
| APS 10% | 0.4 ml |
| TEMED | 30 µl |

***Procedure.*** After cleaning the glass plates and spacers with water and ethanol, the gel apparatus was assembled and the resolving gel solution was poured (app. 10 cm height), and layered with water. The gel was let to polymerize for 3-4 hours or overnight at room temperature.

The layer of water was removed, the stacking gel solution was poured on top of the resolving gel, the comb was inserted and the stacking gel was let to polymerize for 1 hour at room temperature. After removing the comb 1X running gel buffer was added to the chamber and the wells were washed with a syringe. Protein samples were loaded into the wells and the gel was run overnight at 85 volts. The run was stopped before 36 kD prestained marker protein reached approximately the end of the gel 1cm before running out.

## 2.9 Electroblotting and immunodetection of proteins (Western Blot)

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane by semi-dry blotting. Afterwards specific proteins were immunodetected using specific antibodies.

**Materials and solutions**

- Nitrocellulose transfer membrane, cut to the same dimensions as the gel.
- Six pieces of Whatman® 3 MM filter paper, cut to the same dimensions as the gel.
- Blotting chamber.
- Blotting buffer : 25 mM Tris/HCl; 150 mM glycine pH 8.3, 10 % (vol/vol) methanol
- Millipore water

***Procedure*.** The blotting chamber was assembled as follows: Three sheets of filter paper (Whatman® 3MM) are soaked in blotting buffer were placed in the centre of the graphite anode of the blotting chamber. On top of these sheets, equilibrated nitrocellulose membrane was placed. The SDS gel (devoid of stacking gel) was layered on top of the nitrocellulose membrane, avoiding air bubbles. Three sheets of filter paper, presoaked in blotting buffer, were again placed on top of the gel followed by the graphite cathode of the blotting chamber. Transfer was achieved by application of 0.8-0.9 $mA/cm^2$ current for approximately 2 hours.

## 2.10 Ponceau staining of proteins

To estimate the efficiency of protein transfer after blotting, the membrane was stained with Ponceau-S. This stain is reversible and produces pink bands on a light background. The nitrocellulose membrane was washed with Millipore water for 1 minute, incubated in Ponceau-S solution for 2-3 minutes with constant shaking at room temperature. Subsequently the membrane was destained by washing in demineralized water to the desired contrast. Finally it was documented by a digital camera. To remove the stain completely, the membrane was washed again with TBST (1x TBS plus 0.1 % (vol/vol) Tween 20) under constant shaking.

## 2.11 Immunodetection of proteins

### Solutions

**10x Tris-buffered saline (TBS)**

| | |
|---|---|
| Tris/HCl (pH 7.4) | 100 mM |
| NaCl | 1.6 M |

**TBS Tween (TBST)**

1x TBS

0.1 % (vol/vol) Tween 20

**Blocking-buffer and antibody-dilution buffer**

5 % (wt/vol) BSA in 1x TBST (BSA)

**Primary Antibodies**

| Antibody | Dilution | Dil.buffer |
|---|---|---|
| Cx43 (Mouse IgG, polyclonal) | 1:1000 | TBST+BSA |
| PhosphoCx43 Ser368 (Rabbit IgG, polyclonal) | 1:1000 | TBST+BSA |
| Cx37 (Mouse IgG, polyclonal) | 1:1000 | TBST+BSA |
| Cx40 (Rabbit IgG, polyclonal) | 1:1000 | TBST+BSA |
| Tubulin (Rabbit IgG, monoclonal) | 1:2000 | TBST+BSA |
| Vinculin (Clone hVIN-1, mouse IgG) | 1:2000 | TBST+BSA |

**Secondary antibodies, horseradish peroxidase (HRP)-labeled**

| Antibody | Dilution | Dilution buffer |
|---|---|---|
| Anti-mouse IgG | 1:1000 | BSA |
| Anti-Rabbit IgG | 1:1000 | BSA |

***Procedure.*** After a brief washing with Millipore water and TBST, the membrane was blocked with 5% (wt/vol) BSA in TBST for 1 hour at room temperature. After blocking,

the membrane was incubated with primary antibody overnight at 4 °C. The membrane was then washed with TBST 3-4 times for 8-10 minutes each at room temperature and incubated with secondary antibody for 45 min at room temperature. After repeated washing with TBST 3-4 times for 10-15 minutes (each) the membrane was then incubated with enhanced chemiluminescence (ECL) solution and the luminescence was detected and recorded with *Bio-Rad Quantity One* gel documentation system and *ChemiDoc* imaging system.

## 2.12 Immunocytochemistry

Endothelial monolayers on glass cover slips were washed with 1X PBS and fixed with 4 % (wt/vol) paraformaldehyde at 37 °C for 20 min, followed by permeabilization with 0.1 % (wt/vol) Triton X-100 at 37 °C for 20 min. Cells were then washed 3-4 times with 1X PBS and blocked with 5 % (wt/vol) bovine serum albumin and 5 % (wt/vol) cold water fish skin gelatin for 1 h to prevent non-specific binding. Samples were then probed with primary antibody (1:500 dilution) overnight at 4 °C, followed by thorough washing in 1X PBS. Secondary antibodies tagged with Alexa fluor 488/ Alexa fluor 633 were used in a dilution of 1: 250 – 1:500 for 1 h at room temperature. The samples were then washed 3-4 times with 1X PBS and mounted with buffered glycerol. Immunoreactivity was visualized and analyzed by confocal microscopy with a Carl Zeiss LSM 510.

## 2.13 Statistical analysis

Data are given as means ± S.D. of 3-5 experiments using independent cell preparations. The comparison of means between groups was performed by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc test. Changes in parameters within the same group were assessed by multiple ANOVA analysis. Probability (P) values of less than 0.05 were considered as significantly different ($P < 0.05$).

# 3 RESULTS

## 3.1 Effect of reoxygenation on ATP release from endothelial cells

Previously it has been shown that ATP, either exogenously applied or spontaneously released from coronary endothelial cells during reperfusion, protects against reperfusion-induced intercellular gap formation (Gündüz et al., 2006). Pilot experiments were performed to prove whether ATP is released in a similar way under the experimental conditions applied in the present study. Accordingly, cultured endothelial cells from porcine aorta were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$= 140 mmHg). Control cells were exposed to normoxia for the same period of time. Supernatants of cell cultures were collected at indicated time points. ATP concentration of the supernantant was found to be increased after 5 min of reoxygenation with a maximum at 15 min (Fig 1), whereas, no change in the ATP concentration was observed during normoxic and hypoxic conditions (data not shown).

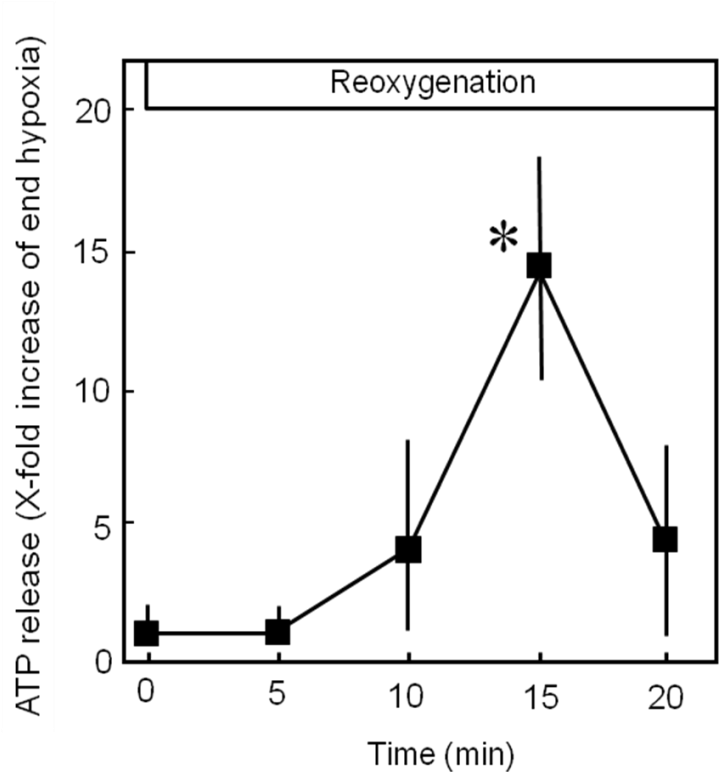



**Figure 1:** Effect of reoxygenation on ATP release from porcine aortic endothelial cells. Cells were exposed to hypoxia ($Po_2$ < 10 mmHg) for 40 min followed by reoxygenation ($Po_2$ = 140 mmHg). ATP concentration of the culture supernatant was determined during reoxygenation at indicated time points. ATP concentrations are given as X-fold of the end-hypoxia value set to 1 (time point 0). Data are means ± SD of five separate experiments of four independent cell preparations. *P < 0.05 vs. time point 0 (end of hypoxia).

As the increase in ATP concentrations in the supernatants of cells exposed to hypoxia and reoxygenation could be due to cell lysis, lactic acid dehydrogenase activity (LDH) was measured as surrogate parameter of cell membrane damage. As shown in Fig 2, LDH activity in the supernatant was below detection limit under all conditions tested, indicating that reoxygenation-induced ATP release from endothelial cells is not due to cell lysis.

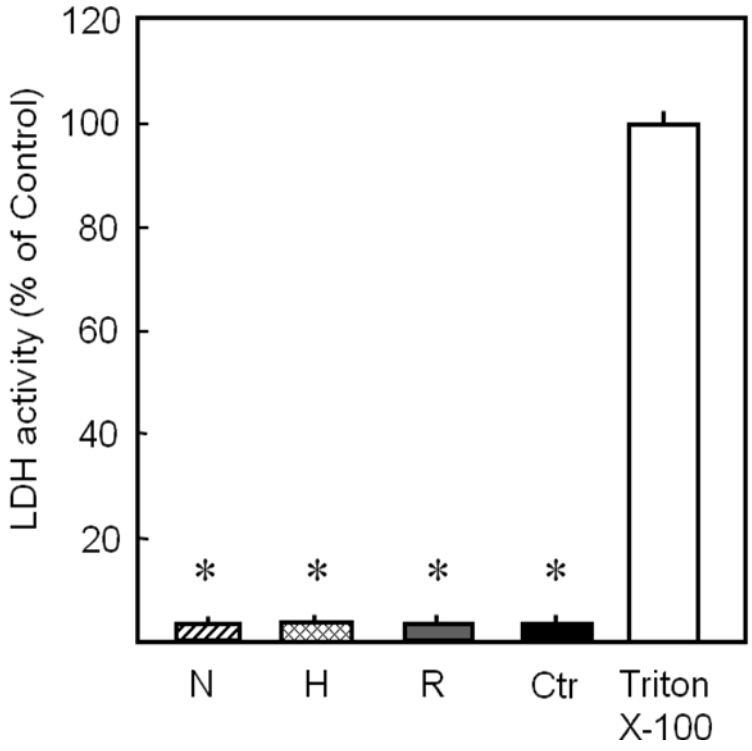



**Figure 2:** The effect of hypoxia and reoxygenation on lactic acid dehydrogenase (LDH) activity in the culture supernatant of endothelial cells. Endothelial cells were exposed to 40 min of normoxia (N), or 40 min of hypoxia (H) followed by 15 min of reoxygenation (R). In a set of experiments cells were collected before start of hypoxia (Ctr). Cells lysed with Triton X-100 served as a positive control and the LDH activity was set to 100%. Data are means of 3 experiments of different cell preparations. *P < 0.05 vs. Triton X-100 control.

## 3.2 Effect of pannexin and connexin channel inhibitors on reoxygenation-induced ATP release from endothelial cells

Pannexin or connexin hemichannels have been shown to be involved in ATP release from endothelial cells (Locovei et al., 2006; Goodenough and Paul, 2003). To analyze whether reoxygenation-induced ATP release is mediated by pannexins or connexins, the effect of pharmacological inhibitors on reoxygenation-induced ATP release was tested. The inhibitors were added at the onset of hypoxia followed by reoxygenation and supernatants were analyzed at 15 min of reoxygenation. The involvement of pannexin was tested by use of probenecid, known to inhibit the opening of pannexin channels (Silverman et al., 2008). This effect was verified by use of mefloquine which has been shown to inhibit pannexins at low concentrations (Dubyak, 2009; Iglesias et al., 2008).

As shown in Fig 3, neither probenecid in a concentration of 0.01 to 1 mM nor mefloquine at concentrations of 0.01 to 1 µM influenced reoxygenation-induced ATP release. However, mefloquine concentrations above 10 µM as well as the pan-specific connexin inhibitors like 18-α glycyrrhetinic acid (GA) or flufenamic acid (FFA) (Goodenough and Paul, 2003) blocked reoxygenation-induced ATP release in a concentration dependent manner. Additionally, lanthanum chloride ($La^{+3}$), which was shown to specifically inhibit the opening of connexin hemichannels but not Gj (Retamal et al., 2007), inhibited reoxygenation-induced ATP release in a concentration dependent manner at 100 and 200 µM.

Taken together, probenecid at any concentration as well as mefloquine at low concentration had no effect on reoxygenation-induced ATP release from endothelial cells speaking against an involvement of pannexins. However, high concentration of mefloquine as well as GA, FFA or $La^{+3}$ were effective to block ATP release indicating that the reoxygenation-induced ATP release is connexin hemichannel dependent.

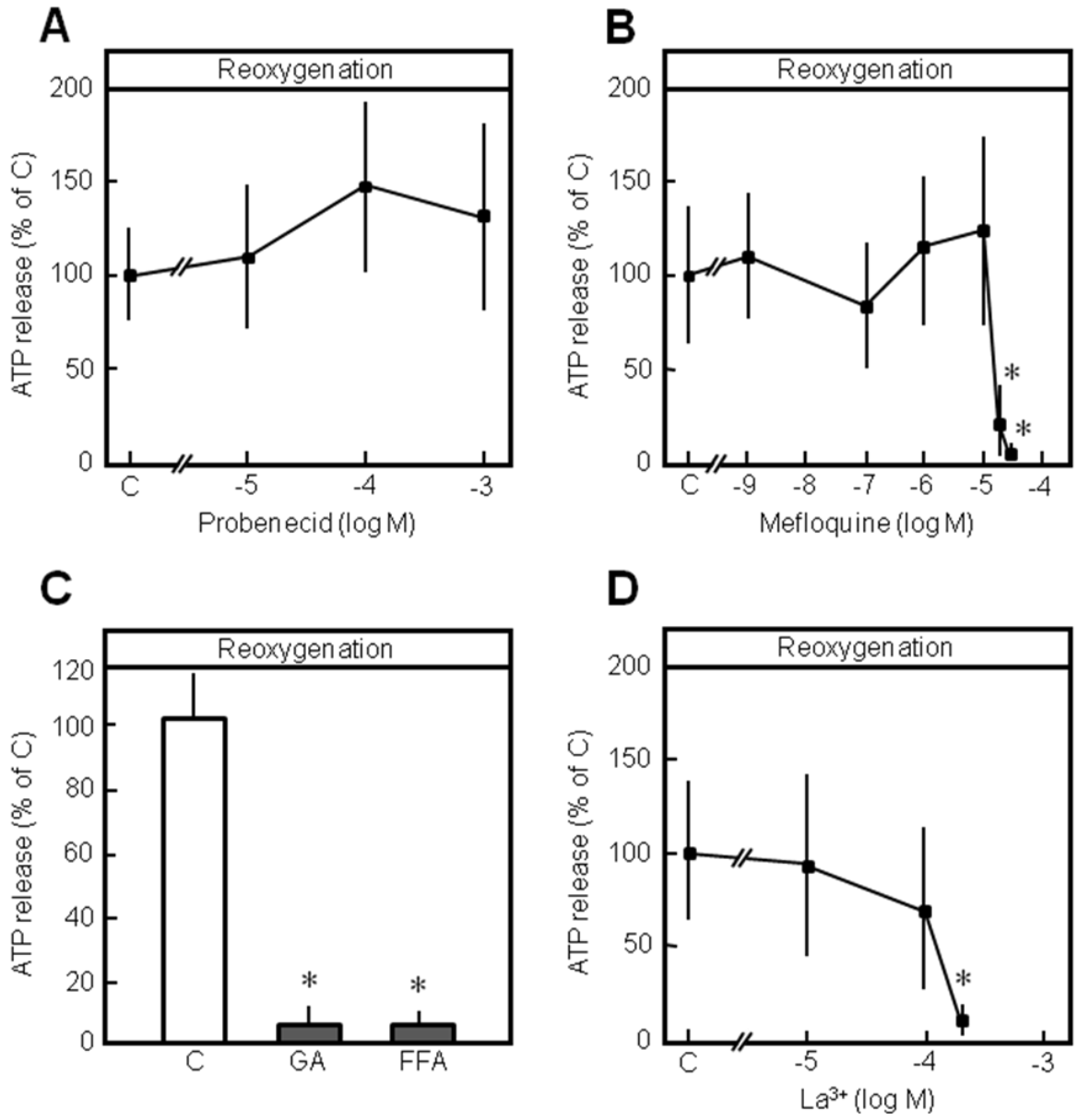



**Figure 3**: Effect of the pharmacological hemichannel inhibitors on reoxygenation-induced ATP release of endothelial cells. Cultured endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). ATP release into the cell culture medium was determined 15 min after onset of reoxygenation, the time point of highest ATP release (see Fig. 1). Each pharmacological agent was added at the onset of hypoxia. (A) Probenecid, a pannexin-specific inhibitor; (B) mefloquine, an agent which blocks pannexin hemichannels at concentrations below 10 µM and connexin hemichannels at concentrations above 10 µM; (C) 18-α glycyrrhetinic acid (GA) or flufenamic acid (FFA), both pan-specific inhibitors of connexins, added at maximum inhibitory concentration of 50 µM; and (D) lanthanum chloride ($La^{3+}$), a connexin hemichannel specific inhibitor. ATP concentrations of the culture supernatants are given as % to control (15 min of reoxygenation). The values of the control were set to 100%. Data are means ± SD of n=3 separate experiments of independent cell preparations. *P < 0.05 vs. control.

## 3.3 Effect of Cx43 silencing on reoxygenation-induced ATP release

*Effect of Cx43 silencing on Cx40 and Cx37 isoforms*

Early studies suggested a role for connexins in reoxygenation-induced ATP release in EC. To verify the data of the pharmacological approach of the preceeding paragraph, endothelial cells were transfected with siRNA specifically targeting Cx43. A significant reduction in the Cx43 protein content was observed after 24 h (upper panel of Fig. 4A), but not in cells treated with non-specific siRNA. Given that various connexins are transcriptionally co-regulated (Isakson et al., 2006; Wang, 2008), the protein content of other connexins such as Cx37 and Cx40 were also determined in Cx43 siRNA treated cells. As shown in the lower panel of Fig 4A, no alterations in Cx37 and Cx40 protein levels was detected in Cx43 siRNA silenced cells compared to untreated controls or cells transfected with non-specific siRNA. Similarly, immunoflourescence staining of Cx43, Cx40, and Cx37 in Cx43 downregulated cells resulted in similar observations as depicted in Fig 4B.

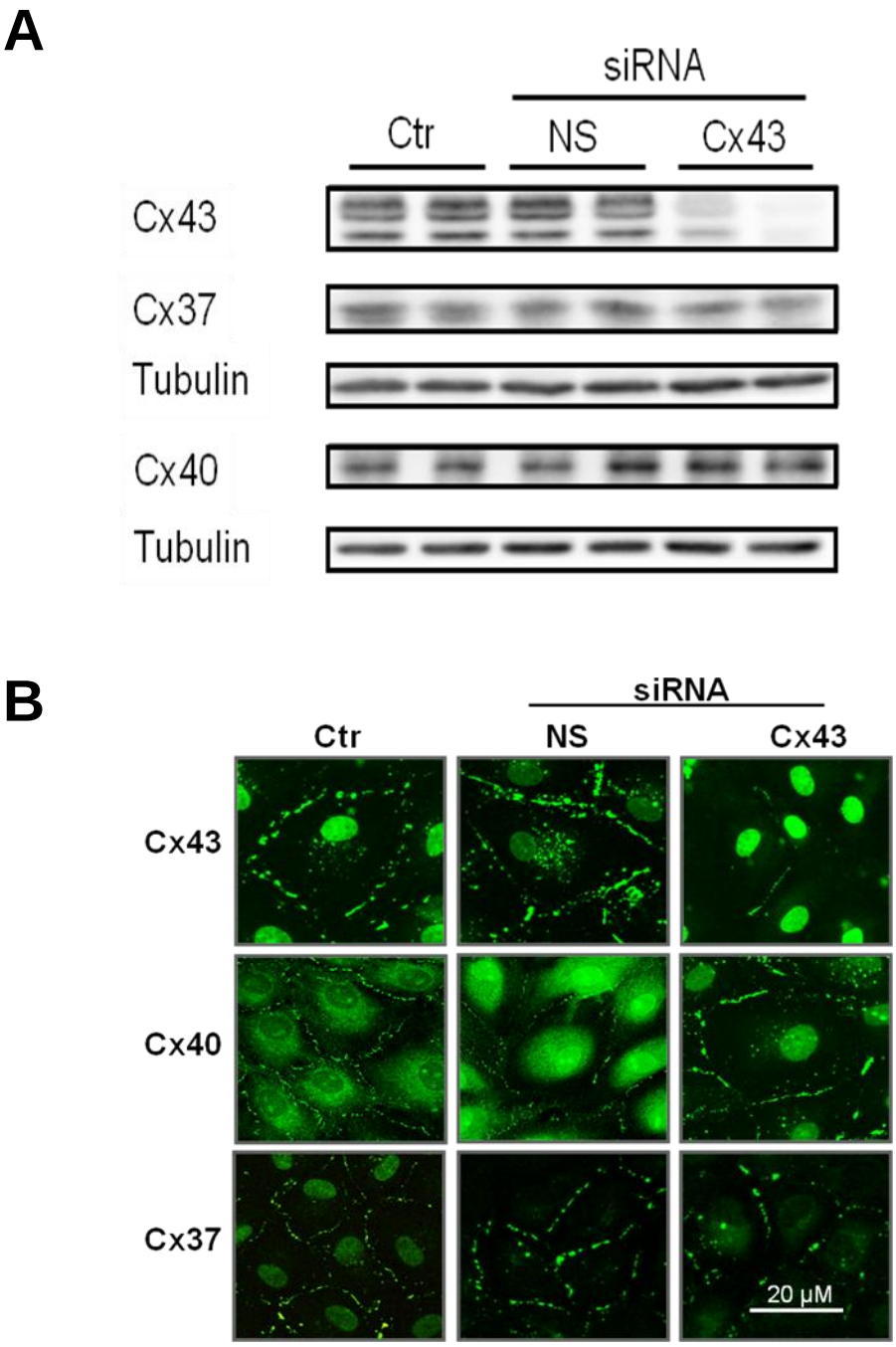



**Figure 4:** Effect of Cx43 silencing on the content of Cx40 and Cx37.
Endothelial cells were transfected with non-specific (NS) or Cx43 siRNA (Cx43) for 24 h under normoxic conditions, while non-transfected cells served as a control (Ctr). **(A)** Representative Western blots of Cx43, Cx40, and Cx37 in endothelial cells. Tubulin was used as respective loading control. **(B)** Immunoflourescence staining of Cx43, Cx40, and Cx37 in endothelial cells.

*Effect of Cx43 silencing on reoxygenation-induced ATP release*

To check whether the induced ATP release in response to reoxygenation in endothelial cells is dependent on Cx43, the ATP concentrations in supernatants of endothelial cells, transfected with non-specific or specific siRNAs of non-transfected cells, were measured 15 min after reoxygenation. As shown in Fig 5, reoxygenation-induced ATP release was abolished in Cx43 down regulated cells. Although siRNA silencing was performed under identical conditions, effectiveness of Cx43 downregulation in endothelial cells varied between experiments. To test whether there is a correlation between Cx43 content and ATP release, the ATP concentration of the culture supernatant was plotted against the corresponding Cx43 content of Cx43 downregulated and control endothelial cells. As shown in Fig 5, ATP release induced by reoxygenation closely correlates with the Cx43 content in endothelial cells.

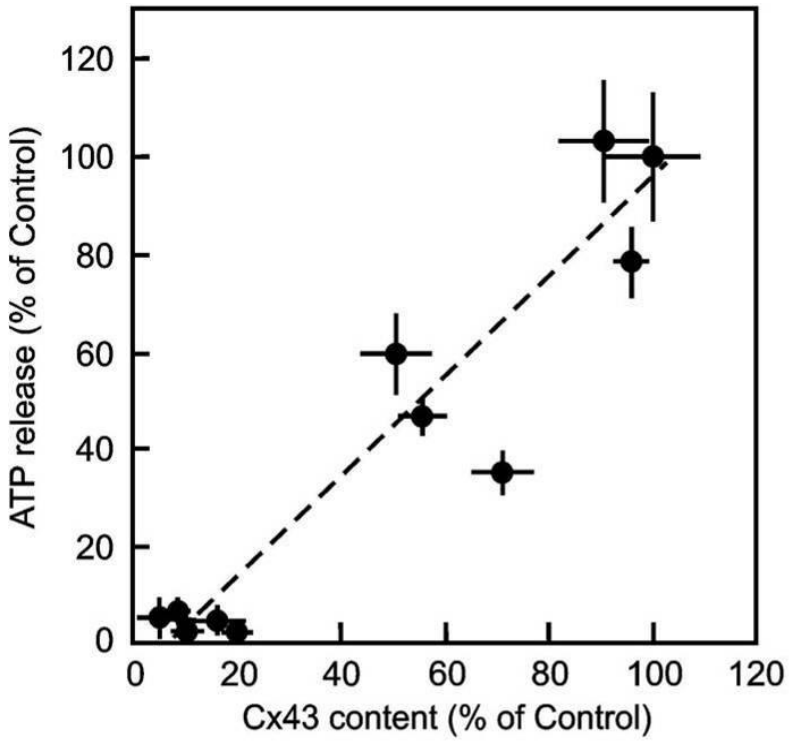



**Figure 5:** Reoxygenation-induced ATP release as function of the endothelial Cx43 content.
Endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). ATP release into the cell culture medium of endothelial cells transfected with specific or non-specific Cx43 siRNA or of non-transfected cells was determined 15 min after onset of reoxygenation, the time point of highest ATP release and Cx43 content of the corresponding cell monolayer was analysed and expressed as percent of untreated controls set to 100%. Data are means ± SD of n = 3 separate experiments of independent cell preparations.

### 3.4 Role of other connexins in reoxygenation-induced ATP release

As shown before, reoxygenation-induced ATP is closely linked to the presence of Cx43 in endothelial cells. Nevertheless, in addition to Cx43, which is one of the major connexin isoforms expressed in endothelial cells, Cx40 and Cx37 may also be involved in ATP release. To test this question, Cx43-downregulated endothelial cells were

additionally exposed to 18-α glycyrrhetinic acid (GA), a pan-specific connexin inhibitor, before onset of hypoxia. As shown in Fig 6, GA did not cause any additional reduction of the reoxygenation-induced ATP release in Cx43-downregulated endothelial cells, supporting the concept that the ATP release is mediated by Cx43.

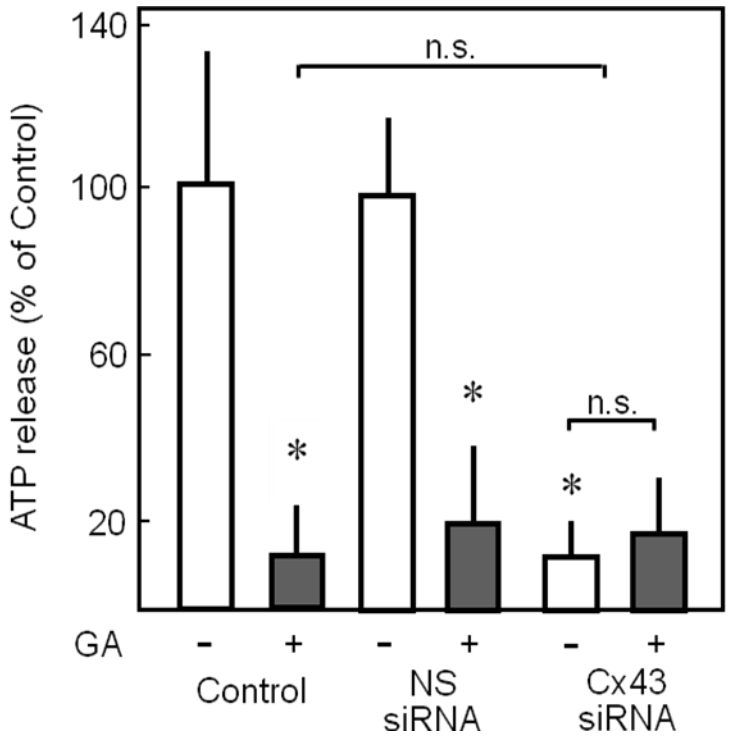



**Figure 6:** Effect of 18-α glycyrrhetinic acid on reoxygenation-induced ATP release in Cx43 silenced cells. Endothelial cells, either transfected with non-specific (NS) or specific Cx43 (Cx43) siRNA as well as untreated control cells, were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). ATP release into the cell culture medium was determined 15 min after onset of reoxygenation, the time point of highest ATP release. ATP release was measured in absence (white bars) or presence (grey bars) of the pan-specific connexin inhibitor 18-α glycyrrhetinic acid (GA; 50 µM) added at the onset of hypoxia. ATP concentrations of the culture supernatants are given as % to control (15 min of reoxygenation). The values of the control were set to 100 %. Data are means ± SD of n=3 separate experiments of independent cell preparations. *P < 0.05 vs. untreated control; n.s.: not significantly different.

### 3.5 Role of PKA in Cx43-dependent ATP release during reoxygenation

As mentioned in the introduction, connexin function is regulated by a variety of kinases directly targeting connexins at specific phoshorylation sites. Activation of PKA is the most important factor for this signaling element, as it is the only kinase known to increase the Cx43 conductivity, transcription, and also channel opening (DeMello, 1988; Lampe and Lau, 2004; Matsumura et al., 2006). In order to investigate the role of cAMP/PKA pathway in the reoxygenation-induced ATP release via Cx43, cells were exposed to four chemically non-related PKA inhibitors blocking PKA by different mechanisms: H-89, KT5720, and two specific protein kinase inhibitor peptides PKI (6-22-amide) or PKI (14-22-amide). As shown in Fig 7A, reoxygenation-induced ATP release was abolished in the presence of all four PKA inhibitors applied. These effects of PKA inhibitors are contrasted by the effect of two different direct PKA activators, i.e. Sp-8-CPT-cAMPs (Sp-8) or 8-Br-cAMPs (8-Br), which both enhanced the release of ATP induced by reoxygenation (Fig 7B). The combined treatment with H-89 plus 8-Br-cAMPs abolished the 8-Br-cAMPs effect (Fig 7C), indicating that the reoxygenation-induced ATP release is mediated by PKA.

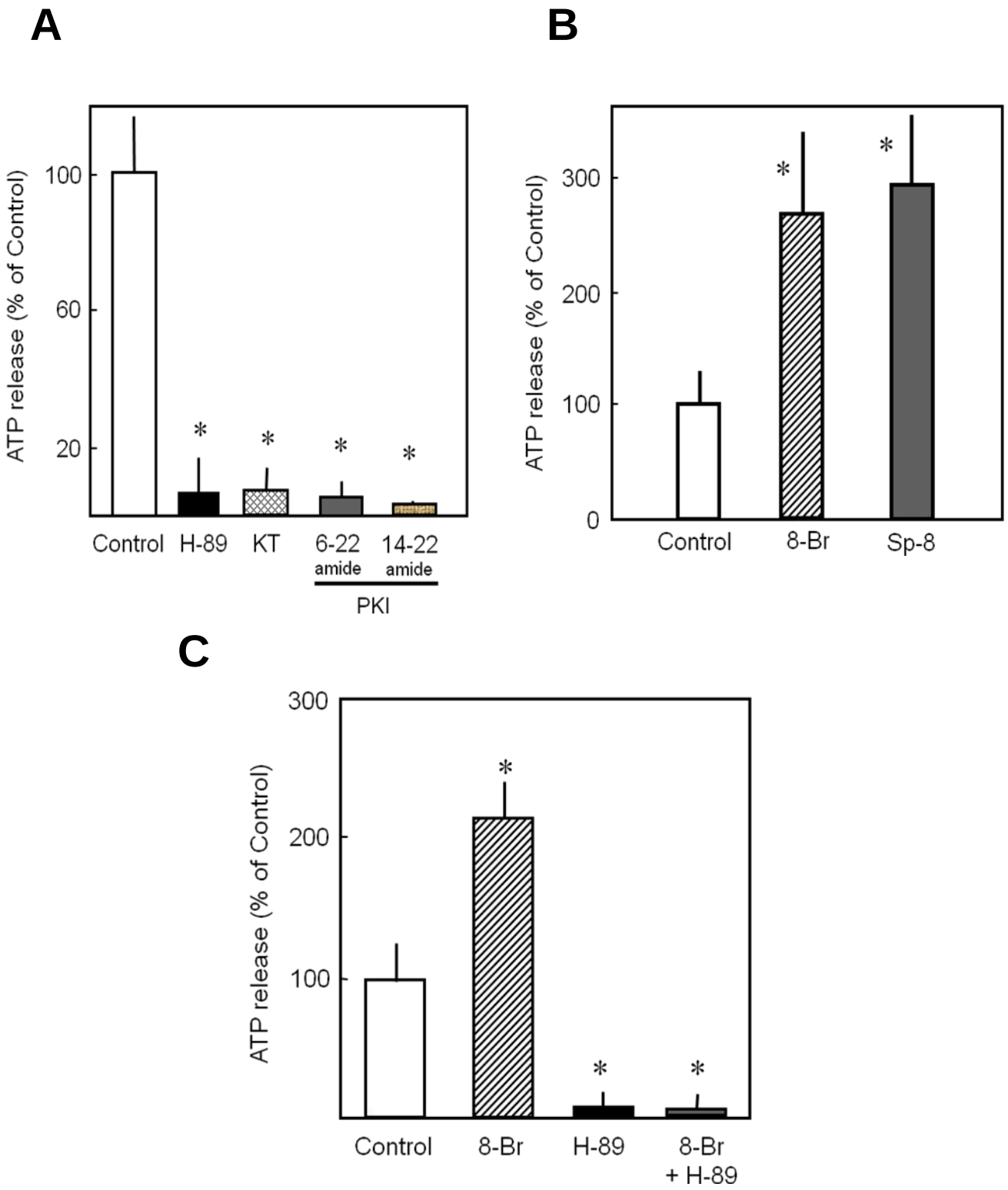



**Figure 7:** Effect of pharmacological inhibition and activation of PKA on the reoxygenation-induced ATP release. Cultured endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). ATP release into the cell culture medium was determined 15 min after the onset of reoxygenation, the

time point of highest ATP release. **(A)** Cells were exposed to PKA inhibitors H-89 (20 µM), KT5720 (KT; 100 nM), or the specific PKA inhibitor peptides (PKI) 16-22 amide (100 nM) or 4-22 amide (5 µM), added at the onset of hypoxia. **(B)** Effect of the PKA activator 8-Br-cAMPs (8-Br; 1 µM) or Sp-8-CPT-cAMPs (Sp-8; 10 µM) on reoxygenation-induced ATP release. The PKA activators were added at the onset of reoxygenation. **(C)** Effect of H-89, 8-Br-cAMPs, and H-89 plus 8-Br-cAMPs on reoxygenation-induced ATP release. ATP concentrations of the culture supernatants are given as % to control (15 min of reoxygenation). The values of the control were set to 100%. Data are means ± SD of n=3 separate experiments of independent cell preparations. *P < 0.05 vs. control.

To prove whether the cAMP/PKA effect on reoxygenation-induced ATP release is mediated by Cx43, cells transfected with Cx43 siRNA or non-specific control siRNA were exposed to hypoxia followed by reoxygenation. 8-Br-cAMPs was added at the onset of reoxygenation. As shown in Fig 8, downregulation of Cx43 in endothelial cells abolished the ATP release induced by reoxygenation. Under this condition 8-Br-cAMPs fails to increase the ATP release, whereas the cAMP analogue enhanced the reoxygenation-induced ATP release in non-transfected or control transfected cells. These findings suggest that the ATP release via Cx43 is modulated by the cAMP/PKA pathway in endothelial cells.

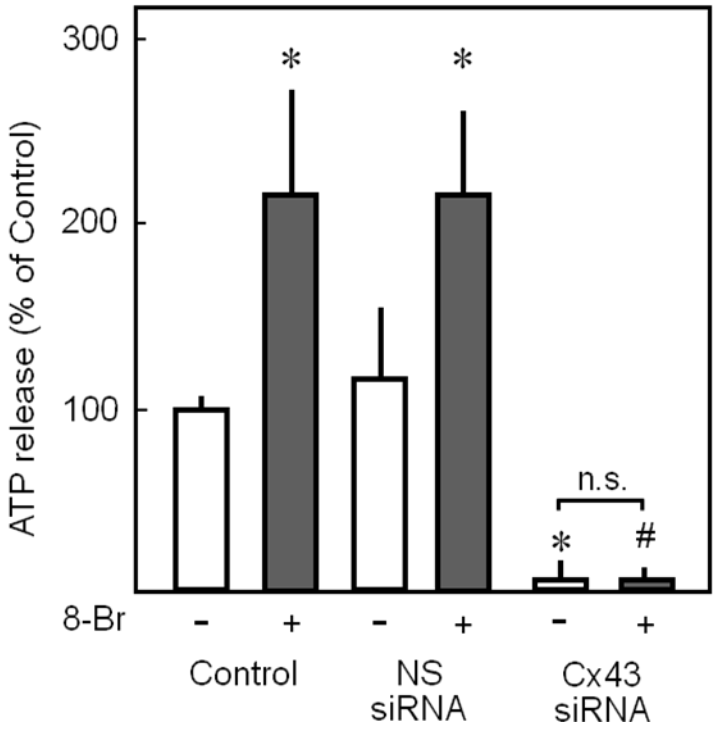



**Figure 8**: Effect of Cx43 silencing on reoxygenation-induced ATP release in presence of the PKA activator 8-Br-cAMPs. Endothelial cells, transfected with non-specific (NS) or specific Cx43 (Cx43) siRNA as well as untreated control cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). ATP release into the cell culture medium was determined 15 min after onset of reoxygenation, the time point of highest ATP release. Cells were exposed to the PKA activator 8-Br-cAMPs (8-Br; 1µM; grey bars), or vehicle control (white bars), added at the onset of reoxygenation, respectively. ATP concentrations of the culture supernatants are given as % of control (15 min of reoxygenation). The values of the control were set to 100%. Data are means ± SD of n=3 separate experiments of independent cell preparations. *P<0.05 vs. respective control. $^{\#}$P<0.05 vs. non-stimulated Cx43 siRNA transfected cells.

## 3.6 Effects of reoxygenation on Cx43 phosphorylation

Gj function is regulated at multiple levels, including Cx43 expression, degradation, and phosphorylation (Duffy et al., 2004; Saffitz et al., 2000; Leithe et al., 2004). The opening propability of Cx43 hemichannels is regulated by phosphorylation of specific serine residues causing a functional change in connexon permeability (Bao et al., 2004). Therefore, the effect of hypoxia/reoxygenation on the phosphorylation state of Cx43 was examined by Western blot analysis. The antibody used reacts with Cx43 when it is phosphorylated at Ser 368. To test the effect of reoxygenation on Cx43 phosphorylation, endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg) and samples were collected at indicated time points. As shown by Westernblot analysis Cx43 migrates in three bands separated during gel electrophoresis (Fig 9 top; Cx43 total). The leading band, indicated as Po, predominantly represents the non-phosphorylated form of Cx43 (Solan and Lampe, 2009; Solan et al., 2007), whereas the two preceding band, indicated as $P_1$ and $P_2$, mainly represent the phosphorylated froms of Cx43 (Musil and Goodenough, 1991). Accordingly, the anti-phospho Cx43 antibody raised against serine 368 detects a band at P1/P2. (Fig 9 top; P~Cx43) which is slightly increased after 5 minutes with a further increase at 15 min of reoxygenation, a time point of maximum ATP release. In contrast, hypoxia alone did not cause an increase in Cx43 phosphorylation or ATP release. This coincidence indicates a causal relationship between Cx43 phosphorylation and ATP release.

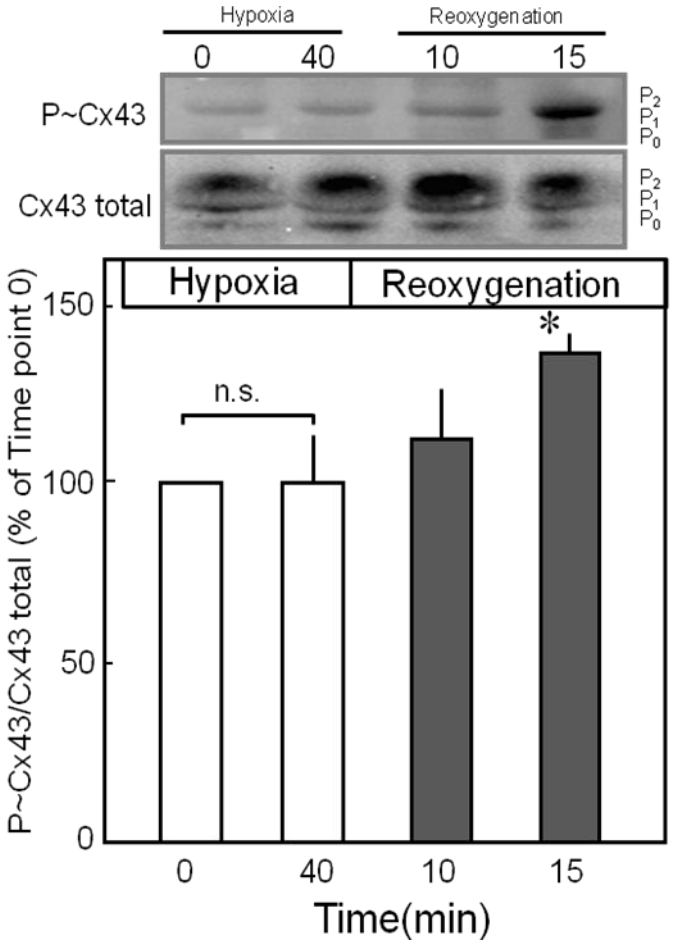



**Fig 9:** Effect of hypoxia and reoxygenation on Cx43 phosphorylation at serine 368. Cultured endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). Representative Western blot of phosphorylated Cx43 (P~Cx43) at Ser 368 (upper panel) in comparison to total Cx43 (lower panel) during hypoxia (0 and 40 min) and reoxygenation (10 and 15 min). $P_2$, $P_1$, and $P_o$ indicate high, low, and non-phosphorylated form of Cx43, respectively. Densitometric analysis of Western blots is shown below. P~Cx43 relative to Cx43 total is given as % of control (0 min) normalized to 100%. Data are means ± SD of n=3 separate experiments of independent cell preparations.*P < 0.05 vs. end of hypoxia (40 min value).

## 3.7 Role of PKA on Cx43 phosphorylation during reoxygenation

Maneuver causing elevation of intracellular cAMP increase Cx43 phosphorylation (Darrow et al., 1995). Therefore it was tested whether those inhibitors or activators of cAMP/PKA pathway as studied in Section 3.5, not only modulated ATP release in endothelial cells but also were able to influence the phosphorylation of Cx43.

The reoxygenation-induced Cx43 phosphorylation was further increased in the presence of PKA activator 8-Br-cAMPs, and this effect could be abolished by the addition of the PKA inhibitors H-89, KT5720 or 6-22-amide peptide (PKI) (Fig 10A), or a combination of H-89 and 8-Br-cAMPs (Fig 10B), indicating that Cx43 may play a role in reoxygenation-induced ATP release modulated by PKA.

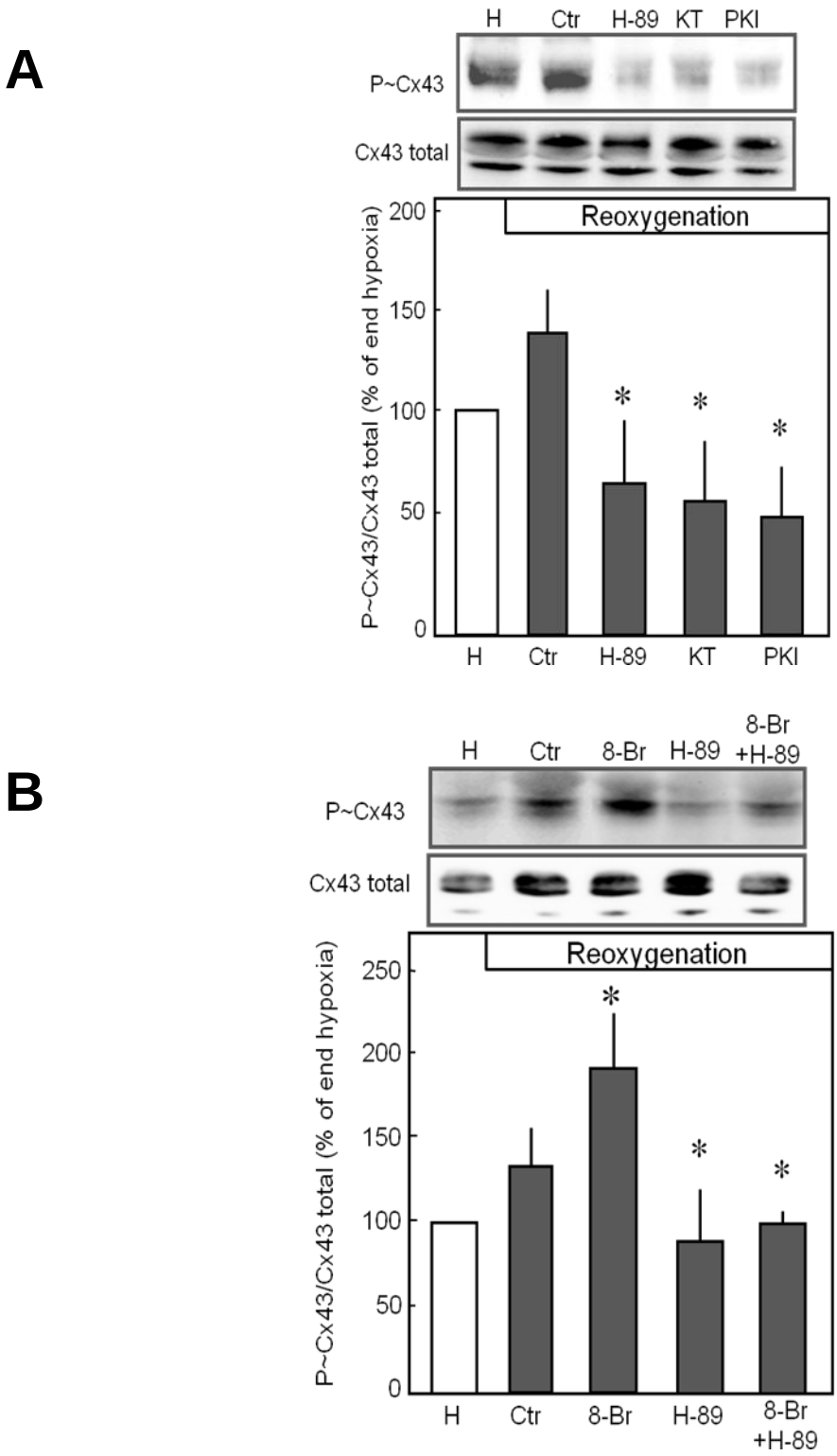



**Fig 10:** Effect of activators and inhibitors of PKA on reoxygenation-induced phosphorylation of Cx43. Cultured endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by 15 min of reoxygenation ($Po_2$ = 140 mmHg). Representative Western blot of phosphorylated Cx43 (P~Cx43) at serine 368 (upper

panel) in comparison to total Cx43 (lower panel) and the densitometric analysis is shown below, respectively. **(A)** Effect of PKA inhibitors H-89 (20 µM), KT5720 (KT; 100 nM) and the specific protein kinase inhibitor peptide (PKI) 6-22 amide (100 nM) during 15 min of reoxygenation, and **(B)** effect of PKA inhibitor H-89, PKA activator 8-Br or H-89 plus 8-Br during 15 min of reoxygenation on Cx43 phosphorylation is shown. (H) Cx43 phosphorylation after 40 min of hypoxia or (Ctr) 15 min of reoxygenation. The values of 40 min end of hypoxia (H) were set to 100 %. P~Cx43 relative to Cx43 total is given as % of control normalized to 100%. Data are means ± SD of n=3 separate experiments of independent cell preparations.*P < 0.05:

### 3.8 Effect of connexin channel openers on reoxygenation-induced ATP release

Connexin channel openers are substances which have the ability to enhance the open propability of connexin channels by stabilizing its phosphorylated form (Clarke et al., 2009; Plotkin, 2002). Two connexin channel openers: anti-arrhythmic peptide 10 (AAP10) and alendronate were used to test whether they can effect reoxygenation-induced ATP release. Therefore, endothelial cells were exposed to hypoxia followed by reoxygenation and the connexin channel openers were added at the onset of reoxygenation. As shown in Fig 11, both connexin channel openers enhanced the ATP release during reoxygenation. Pretreatment of cells with Cx43 siRNA abolished the effect of AAP10 as well as alendronate on ATP release, strengthening the concept that connexin channel opening is associated with ATP release during reoxygenation.

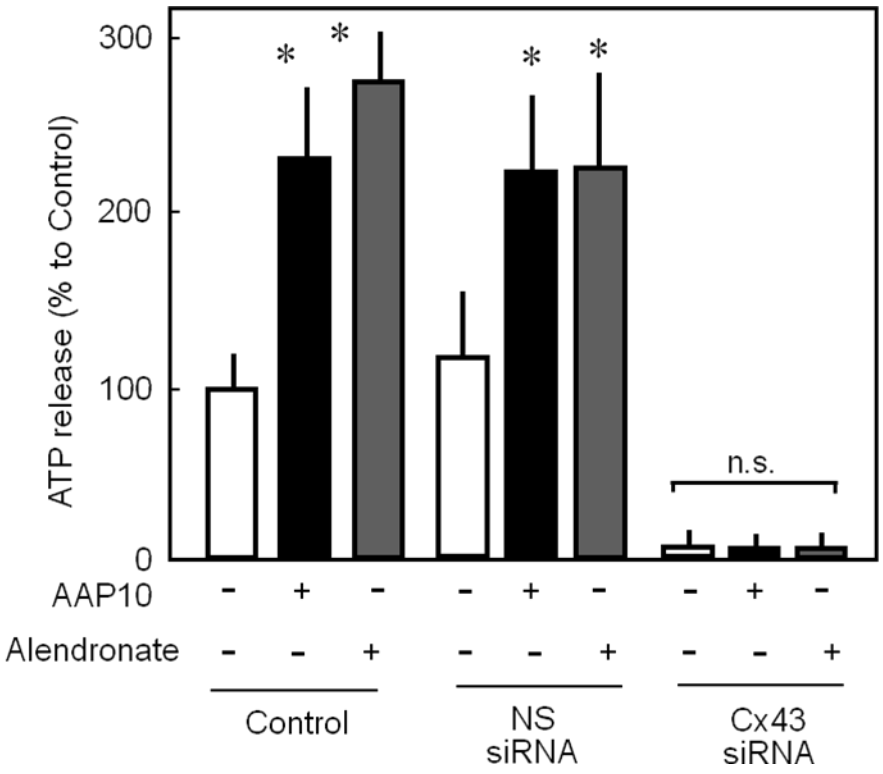



**Figure 11:** Effect of AAP10 and alendronate on reoxygenation-induced ATP release. Cultured endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). ATP release into the cell culture medium was determined 15 min after onset of reoxygenation, the time point of highest ATP release. Endothelial cells, transfected with non-specific (NS) or specific Cx43 (Cx43) siRNA as well as untreated control wells were exposed to AAP10 (10 µM) or alendronate (10 µM). Both agents were added at the onset of reoxygenation. ATP concentrations of the culture supernatants are given as % to control (15 min of reoxygenation). The values of the control were set to 100 %. Data are means ± SD of n=3 separate experiments of independent cell preparations. $^{*}P < 0.05$ vs. control. n.s: not significantly different.

In the next step it was tested whether AAP10 enhances the reoxygenation-induced ATP release via the PKA pathway. For that reason, endothelial cells were pre-incubated with the PKA inhibitor H-89 prior to hypoxia. AAP10 was added at the onset of reoxygenation. As shown in Fig 12, H-89 abolished the reoxygenation-induced ATP release even in presence of AAP10 below control level, indicating that the enhanced ATP release induced by AAP10 is triggerd by PKA.

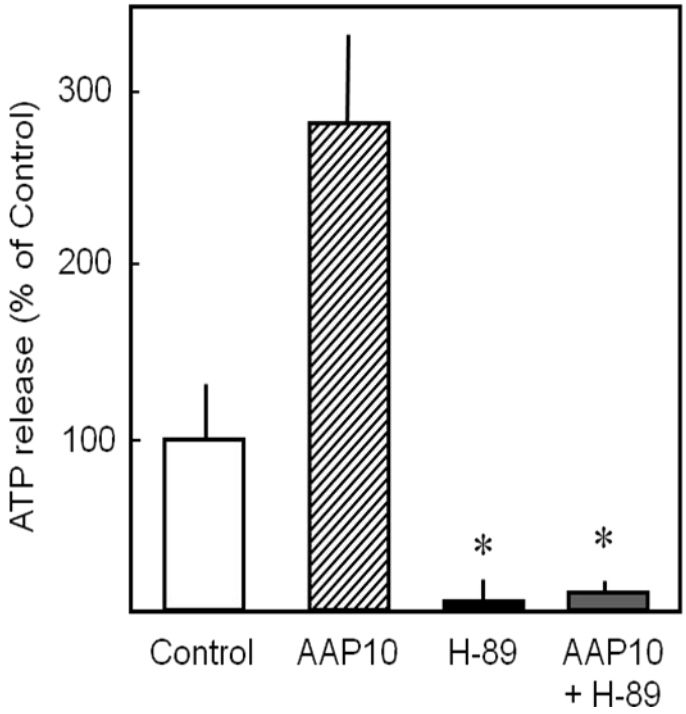



**Figure 12:** Effect of the connexin channel opener AAP10 and the PKA inhibitor H-89 on reoxygenation-induced ATP release. Cultured endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by reoxygenation ($Po_2$ = 140 mmHg). ATP release into the cell culture medium was determined 15 min after onset of reoxygenation, the time point of highest ATP release. The PKA inhibitor H-89 (20 µM) was added at the onset of hypoxia and AAP10 (10 µM) at the onset of reoxygenation. ATP concentrations of the culture supernatants are given as % to control (15 min of reoxygenation). The values of the control were set to 100%. Data are means ± SD of n=3 separate experiments of independent cell preparations. *P<0.05 vs. control.

Finally, it was tested whether AAP10 affects Cx43 phosphorylation. Addition of AAP10 at the onset of reoxygenation increased Cx43 phosphorylation. However, the same effect is abolished after pre-incubation with H-89 prior to reoxygenation even in the presence

of AAP10 (Fig 13). PKA inhibition by H-89 reduced the Cx43 phosphorylation indicating that during reoxygenation PKA cause a Cx43 phosphorylation.

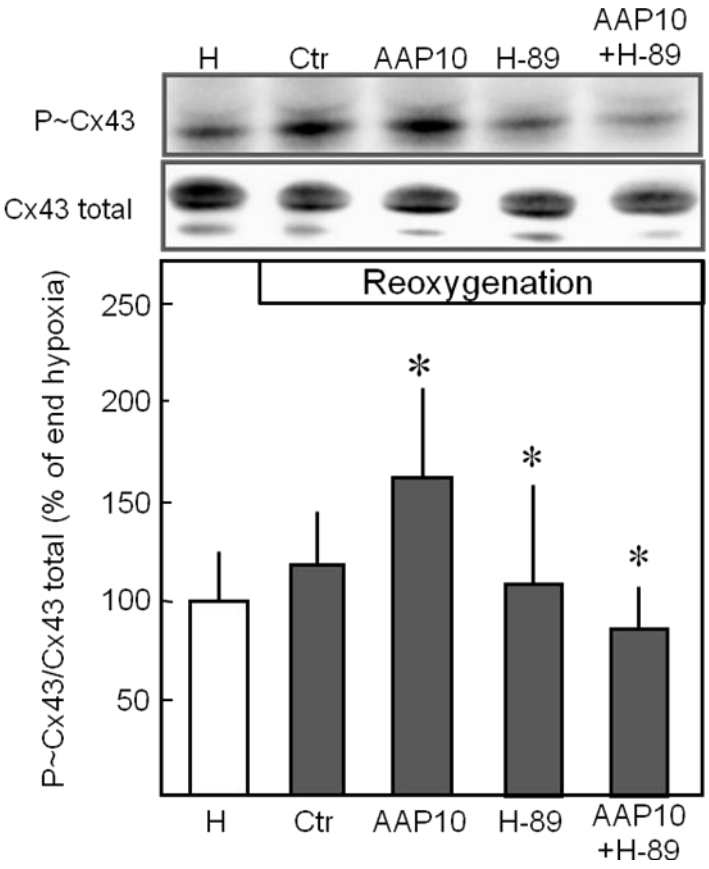



**Fig 13:** Effect of AAP10 and the PKA inhibitor H89 on reoxygenation-induced Cx43 phosphorylation. Cultured endothelial cells were exposed to 40 min of hypoxia ($Po_2$ < 10 mmHg) followed by 15 min of reoxygenation ($Po_2$ = 140 mmHg). Representative Western blots of phosphorylated Cx43 (P~Cx43) at Ser 368 (upper panel) in comparison to total Cx43 (lower panel). (H) Cx43 phosphorylation after 40 min of hypoxia or (Ctr) 15 min of reoxygenation. H-89 (20 µM) was added at the onset of hypoxia, whereas AAP10 (10 µM) at the onset of reoxygenation. A representative experiment of 3 is shown. The values of 40 min end of hypoxia (H) were set to 100%. P~Cx43 relative to Cx43 total is given as % of control set to 100 %. Data are means ± SD of n=3 separate experiments of independent cell preparations.*P < 0.05 vs. C.

# 4 DISCUSSION

## 4.1 Main findings

Protection of endothelial barrier function is inevitable for the recovery of the reperfused heart and I-R causes failure of the endothelial barrier. The consequences are myocardial edema and functional impairment of the myocardium (Mehlhorn et al., 2001). ATP has been proven to be a barrier protective agent due to its presence in the surrounding environment of EC (Gündüz et al., 2006). As shown by Schwiebert et al., (2002) the endothelium is the local source of ATP within the vascular wall. EC release ATP under basal conditions. Enhanced release of ATP from endothelial cells was observed in response to various stimuli including hypotonic challenge, calcium agonists, shear stress, thrombin, ATP itself, lipopolysaccharides, and conditions of I-R (Schwiebert et al., 2002; Pearson and Gordon, 1979; Bodin and Burnstock, 1998; Bergfeld and Forrester, 1992; Clemens and Forrester, 1981). It has been shown that extracellular ATP either exogenously applied or spontaneously released from the EC provides protection of endothelial barrier function against reperfusion injury (Gündüz et al., 2006).

In the present study, the hypothesis was tested whether connexins are candidates mediating the release of ATP during reoxygenation. The major findings are: (1) Porcine aortic endothelial cells release ATP during reoxygenation in a Cx43-dependent manner. (2) This reoxygenation-induced ATP release is dependent on Cx43 but not on Cx40, Cx37, or pannexins, and (3) is triggerd by intracellular signal transduction mechanism involving the cAMP/PKA pathway. (4) The ATP release coincided with cAMP/PKA-dependent phosphorylation of Cx43 at Serine 368. (5) Connexin channel openers further enhanced the reoxygenation-induced ATP release and increased Cx43 phosphorylation in a PKA-dependent manner.

## 4.2 Endothelial cells release ATP during reoxygenation via connexins

In the present study, it has been demonstrated for the first time that ATP released during reoxygenation in porcine aortic endothelial cells is Cx43 dependent. This finding implicates a novel function of Cx43 beyond Gj communication in the regulation of

endothelial barrier function. A transient ATP release with the highest ATP concentration in the cell culture medium was observed 15 minutes after onset of reperfusion. Simultaneous determination of LDH activity in the cell culture medium revealed no detectable LDH activity, neither during hypoxia nor during reoxygenation, verifying that the increase in ATP concentration of the culture supernatants was not due to cell lysis. The findings of the present study are in accordance with previous data showing that Cx43 in the plasma membrane is a prerequisite for ATP release from activated PMNs (Eltzschig et al., 2006). Faigle et al. (2008) studied the molecular mechanisms of endothelial-dependent ATP release during hypoxia and suggested a functional role of Cx43 in endothelial ATP release. Another finding suggested that exposure to acute hypoosmotic stress or mechanical stimuli results in the release of intracellular ATP via connexin hemichannels in bovine corneal endothelial cells (Gomes et al., 2005). Similar findings are reported by Stout et al. (2002) and Leybaert et al. (2003) for astrocytes.

Since there is extensive evidence that not only Cx hemichannels but also Panxs channels may be involved in the release of ATP (Locovei et al., 2006; Goodenough and Paul, 2003; Evans et al., 2006; Kang et al., 2008), a pharmacological approach was applied in the present study to identify the routes of ATP release in endothelial cells. The Panxs inhibitor probenicid did not affect the ATP release during reoxygenation, indicating that Panxs channels are not involved in this release process. Mefloquine, which is known to inhibit Panxs at concentration below 10 µM and Cx at concentrations above 10 µM (Dubyak, 2009; Iglesias et al., 2008), did not have any effect on reoxygenation-induced ATP release at low concentrations. On the contrary, at concentrations above 10 µM, mefloquine reduced the reoxygenation-induced ATP release. The data further support the concept that Cx, rather than Panxs, channels are involved. In line with these data the presence of 18-α glycyrrhetinic acid (GA, 50µM) or flufenamic acid (FFA, 50µM), two pan specific inhibitors of Gj and Cx hemichannels, abolished reoxygenation-induced ATP release. Similar results were obtained with lanthanum chloride, a more specific Cx hemichannels blocker (Retamal et al., 2007), strengthening the conclusion that ATP is released via Cx hemichannels. The data of the present study are in line with a report from Faigle et al (2008), who have studied the molecular mechanisms of ATP release from endothelial cells during hypoxia. The

authors found that this release was abolished by using pan-specific inhibitors of Gj. Furthermore, connexin-mediated ATP release does not occur in endothelial cells only. Clarke et al., (2009) showed that the ATP release from myocytes under ischemia was attenuated in the presence of the connexin channel inhibitor glycyrrhetinic acid, suggesting that ATP release in a connexin-dependent manner is a mechanism not only restricted to endothelial cells but rather ubiquitous for all cells of the cardiovascular system.

## 4.3 Reoxygenation-induced ATP release is predominantly mediated via connexin 43

Since pharmacological inhibitors are quite unspecific in their mode of action (Evans and Boitano, 2001), reoxygenation-induced ATP release was determined in cells in which the Cx43 content was reduced by Cx43 siRNA transfection. In Cx43 silenced cells, release of ATP closely correlated with the cellular Cx43 content, suggesting that reoxygenation-induced ATP release is mediated via Cx43 hemichannels. There is evidence that reduction in Cx43 expression may lead to co-regulation of the expression of many other proteins, e.g. connexins too. Wang et al., 2008 reported that downregulation of Cx43 by siRNA alters the expression profile of human aortic endothelial cells in culture. They showed that Cx43 downregulation caused an increase in mRNA levels of Cx37 and Cx40, whereas the protein level of both connexins did not change in that model. Moreover, Cx40-/- mice have been demonstrated to have decreased Cx37 and Cx43. (Isakson et al., 2006; Simon and McWorter, 2003). This and other findings that the expression of several other proteins is disturbed in hearts from Cx43 double kockout mice had led to the conclusion that the expression of connexins are coregulated (Iacobas et al., 2005). In contrast, the present study showed that Cx43 silencing did not affect the expression of Cx37 and Cx40, indicating that the reduction of ATP release in Cx43 silenced cells is due to lack of Cx43, but not of Cx37 or Cx40. In line with that, exposure of Cx43-silenced endothelial cells to the pan-specific connexin inhibitor, glycyrrhetinic acid, did not cause a further reduction of the ATP release during reoxygenation, supporting the concept that ATP release from endothelial cells is mediated by Cx43. A similar mechanism has also been described by Eltzschig et al. (2006) for PMN. In an animal model of inducible Cx43-deficient mice they showed that

Cx43 expression correlate with ATP release from PMN. Furthermore, the study demonstrate liberation of nucleotides at sites of acute inflammation by PMN, and identify Cx43-dependent ATP release which later on control the adenosine-dependent endothelial function.

## 4.4 Role of PKA in reoxygenation-induced ATP release

It is well documented that maneuvers increasing cellular cAMP levels in endothelial cells protect against endothelial barrier dysfunction in reponse to proinflammatory mediators like histamine, thrombin, oxidants, and tumor necrosis factor (Baluk and McDonald, 1994; He and Curry, 1993; Ochoa et al., 1997; Patterson et al., 1994; Sheldon et al., 1993). The protective effect of cAMP on endothelial barrier function has been studied *in vitro* and in intact organ models (Minnear et al., 1989; Ochoa et al., 1997) and under I-R conditions (Adkins et al., 1992). The mechanism by which cAMP functions to regulate endothelial permeability is presumed to occur predominantly through activation of PKA and the cAMP-binding protein Epac (exchange protein directly activated by cAMP) leading to stabilization of the endothelial cytoskeleton as well as cell-cell and cell-matrix adhesion structures (Cullere et al., 2005; Sayner, 2011).

Surprisingly, direct activation of PKA by two different cAMP analogue, Sp-8-CPT as well as 8-Br-cAMPs, enhanced the ATP release during reoxygenation. These effects were abolished by combined treatment of the endothelial cells with a PKA activator plus inhibitor, 8-Br-cAMPs plus H-89, indicating that reoxygenation-induced ATP release is PKA mediated. These data were verified by application of a chemically not related peptide PKA inhibitor, PKI. PKI is a more specific inhibitor of PKA than H-89 or KT5720, which unlike PKI, act on other protein kinases and the cytosolic $Ca^{2+}$ level as well (Davies et al., 2000). PKI binds to the free catalytic subunit of PKA and prevents phosphorylation of PKA targets (Murray, 2008). Similar function of cAMP/PKA was reported in rabbit erythrocytes which results in stimulation of signaling pathways that culminate in ATP release, indicating that cAMP/PKA-mediated ATP release may be a ubiquitous mechanism in cells of the cardiovascular system (Adderley et al., 2010).

Depending on the cell type, there are several pathways by which ATP may be released, e.g. via ATP-binding cassette (ABC) proteins, vesicles, as well as Cx or Panxs hemichannels (Goodenough and Paul, 2003; D'hondt et al., 2009). Evidence for an involvement of Panxs in ATP release comes from erythrocytes (Sridharan et al., 2010). The authors show that erythrocytes, when exposed to hypoxia, release ATP via Panxs channels. On the basis of several studies, Cx channels have been proposed to mediate ATP release in endothelial cells (Goodenough and Paul, 2003; Faigle et al., 2008; Gomes et al., 2005). In the present study, the involvement of Cx43 in the ATP release during reoxygenation was tested considering that other Cx as 43, and Panxs, may be involved in this liberation process. As mentioned above, Cx inhibitors interfere with ATP release. In addition, there is a notable correlation between Cx expression and ATP release. The involvement of Cx43 in ATP release has also been tested in Cx43 silenced endothelial cells challenged by PKA activators to enhance ATP release at the onset of reperfusion. In these experiments reoxygenation-induced ATP release was abolished even in the presence of PKA activators, indicating the functional impact of Cx43 on reperfusion-induced ATP release. Thus, it was reasonable to infer that Cx provided the ATP release pathway in the form of Cx hemichannels in the non-junctional membrane.

## 4.5 Phosphorylation of Cx43 during reoxygenation is regulated by cAMP/PKA pathway

Cx hemichannel as well as Gj activity is regulated at multiple levels, including Cx43 expression, degradation, and phosphorylation (Duffy et al., 2004; Saffitz et al., 2000; Leithe et al., 2004). From the literature it is known that hypoxia or ischemia effect phosphorylation state of Cx43 in cardiac cells (Beardslee et al., 2000; Turner et al., 2004). As a result of this, change in the phosphorylation status of serine residues of Cx43 hemichannels ultimately leads to the functional change in connexon permeability (Bao et al., 2004).

As identified by Western blot analysis, phosphorylation of Cx43 at Ser368 was increased during reoxygenation in endothelial cells. This phosphorylation can be enhanced when the cells were reperfused in presence of direct PKA activator, and blunted in presence of three chemically not related PKA inhibitors. The coincidence of

the alterations of Cx43 phosphorylation and Cx43-mediated ATP release induced under identical conditions strongly suggests a causal relationship between ATP liberation and Cx43 in endothelial cells.

Until today, there are no data available on Cx43 phosphorylation during the reperfusion conditions. Up to eight serine residues on the carboxyl tail of Cx43 are known to be phosphorylated by different kinases (Solan et al., 2007), but their relationship to hemichannel gating remains unclear (Sáez et al., 2005). It is well known that serine368 (Ser368) is the PKC-dependent phosphorylation site of Cx43, whereas serine364 (Ser364) is targeted by PKA (Lampe et al., 2000; Ek-Vitorin et al., 2006; TenBroek et al., 2001). However, it has also been reported that PKC and PKA may phosphorylate common serine residues (Wecker et al. 2001). Moreover, Yogo et al. (2006) have found that PKA has no selectivity among the four serine residues Ser365, Ser368, Ser369, and Ser373, indicating that these four serine residues were actually target sites for PKA.

### 4.6 Connexin channel opener enhances the reoxygenation-induced ATP release

During inflammation, failure of endothelial barrier is induced by a plethora of mediators leading to the edema formation. On the other hand, mediators like ATP, which may induce opposing effects on barrier function, are also released from vascular cells in response to pathophysiological conditions (Bodin and Burnstock, 1998; Bergfeld and Forrester, 1992; Clemens and Forrester, 1981; Bodin and Burnstock, 2001). It has been shown that extracellular ATP protects the endothelial barrier against the injurious effect of an inflammatory mediators such as thrombin (Gündüz et al., 2003; Kolosova et al., 2005; Härtel et al., 2007). In addition, it has been shown that ATP is released from endothelial cells during reperfusion and blunts reperfusion-induced barrier failure (Gündüz et al., 2006).

The present study revealed that ATP is released from endothelial cells in response to reoxygenation via a Cx43-dependent mechanism and that this release can be enhanced by maneuver activating the PKA signaling pathway targeting Cx43 phosphorylation in endothelial cells. These data not only provide a molecular basis of

ATP release, but also point to a therapeutic option to enhance the accumulation of ATP in the extracellular medium and its barrier protective effect. However, due to its ubiquitous presence in all cells and involvement in countless signaling mechanisms mediating conflicting cellular effects, activation of the cAMP/PKA pathway is a less promising strategy for endothelial protection.

Recently, it has been shown that Cx function, in regard to Gj communication, can be enhanced by a group of substances known as Cx channel openers (Clarke et al., 2009; Plotkin et al., 2002). Therefore, two pharmacological non-related Cx channel openers were tested in the present study and their effect on reoxygenation-induced ATP release as well as their influence on cAMP/PKA triggered regulatory signaling on Cx43 was analyzed.

Alendronate, a 250 Da synthetic molecule, belongs to the group of bisphosphonates. It is applied for the treatment of several bone diseases and shows anti-apoptotic effects on osteocytes and osteoblasts by opening Cx43 hemichannels (Plotkin et al. 1999). This unsuspected role of Cx43 in regulation of cell survival in response to alendronate elucidated a novel Cx function in addition to its well known role in Gj communication (Plotkin et al., 2008).

The second Cx channel opener is the antiarrhythmic peptide AAP10 (Salameh and Dhein, 2005). This is a hexapeptide known to increase intercellular Gj communication (Kjølbye et al., 2003). A much more stable chemical analogue of the hexapeptide AAP10, ZP123, also promoted electrical coupling in ventricular myocytes and attenuated gap junctional closure in acidosis (Eloff et al., 2003; Haugan et al., 2005). Besides preventing arrhythmias, both substances also showed reduced infarct size when applied during and after myocardial infarction (Haugan et al., 2006; Hennan et al., 2006)

Application of both Cx channel openers at the onset of reoxygenation enhanced the ATP release. These effects of AAP10 and alendronate was abolished in Cx43 downregulated cells, demonstrating that Cx43 hemichannel opening during reoxygenation is involved in the ATP release. The results obtained from this study are in

agreement with previous findings (Clarke et al., 2009; Stahlhut et al., 2006), showing a three-fold increase in the release of ATP from myocytes in the presence of AAP10. Based on the observation it seems likely that release of ATP from endothelial cells during reoxygenation could be further enhanced by Cx channel openers acting not only on endothelial cells but also on other cells of the cardiovascular system.

AAP10 was shown to activate PKC in a G-protein dependent manner resulting in Cx43 phosphorylation and improvement of Gj conductance (Weng et al., 2002; Müller et al., 1997; Dhein et al., 2001). As shown in this study, endothelial cells release ATP during reoxygenation via Cx43 hemichannels mediated by PKA activation. This raised the question by which mechanism a Cx channel opener enhances the reoxygenation-induced ATP release. It was shown previously that AAP10 enhances the metabolic coupling via Cx43 Gj through activation of PKC (Easton et al., 2009). Using a pharmacological as well as siRNA approach, it was demonstrated in the present study that the ATP release from endothelial cells in response to reoxygenation is triggered by a PKA-dependent pathway. For that reason, endothelial cells were pre-incubated with H-89 prior to hypoxia and then AAP10 was added at the onset of reoxygenation. There was a tremendous decrease in release of ATP with H-89 plus AAP10 as compared to the controls, suggesting that enhanced ATP release by AAP10 via Cx43 is PKA dependent. In addition, Cx43 phosphorylation was increased when AAP10 was added alone during onset of reoxygenation. Accordingly, the AAP10 effect is abolished when cells are pre-incubated with H-89 prior to reoxygenation even in the presence of AAP10.

So far, the data of the present study show for the first time that AAP10 can modulate Cx43 function in a PKA dependent manner. It enhances ATP release from endothelial cells using the same signaling pathway, which is already stimulated during onset of reoxygenation. Although, the detailed mechanism underlying these effects are not fully understood, maneuver targeting hemichannel opening may be a promising strategy to protect endothelial cells during reperfusion.

## 4.7 Conclusions

The present study demonstrates that reoxygenation of hypoxic endothelial cells liberates ATP in a Cx43-dependent process. The involvement of other Cxs, namely Cx37 and Cx40 or Panxs was ruled out by using pharmacological and molecular approaches. Reoxygenation stimulated the cAMP/PKA signaling pathway which targets Cx43 at Ser368 triggering an ATP release via Cx43. This reoxygenation-induced ATP release can by enhanced by Cx channel openers which also target the cAMP/PKA pathway by an as yet unidentified mechanism. It is suggested that the increase of this selective and temporary opening of Cx43 hemichannels strengthens the protective effect on endothelial barrier function, thus keeping reperfusion injury at bay.

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# 6 SUMMARY

Reperfusion-injury impairs endothelial barrier function and may lead to edema formation, impeding recovery of the reperfused heart. Recently is has been shown that ATP, spontaneously released from endothelial cells (EC) during reperfusion protects the endothelial barrier against reperfusion-injury. The aim of the present study was to identify the mechanism of this spontanous ATP release during reoxygenation in endothelia cells. The central hypothesis is that EC release ATP via a connexin-mediated route. It is shown that macrovascular endothelial cells from porcine (PAEC) release ATP via Cx43 hemichannels at the onset of reperfusion. Determination of LDH activity in the culture medium rouled out that the increase in ATP is due to release from lysed cells. Pharmacological inhibitors of connexin function, i.e., mefloquine at concentrations above 10 µM, 200 µM $La^{3+}$, 50 µM 18-α glycyrrhetinic acid or 50 µM flufenamic acid reduced reperfusion-induced ATP release. In contrast, maneuvers, which inhibit pannexins, like probenecid at concentrations between 10 µM to 1 mM or mefloquine at concentrations below 10 µM, did not affect ATP under the same conditions, indicating that ATP release is mediated via connexins rather than pannexins. These results were confirmed by using a specific Cx43-siRNA transfection strategy. The addition of the connexin hemichannel openers i.e anti-arrythmic peptide 10 (AAP10, 10 µM) or alendronate (10 µM) significantly increased ATP-release compared to the reoxygenated control. Reoxygenation-induced ATP release was also enhanced when a specific PKA-activator like 8-BrcAMP (1 µM), and Sp8CPT (10 µM) was added with the onset of reperfusion, while H-89 (20 µM), KT-5720 (100 nM) as well as two specific protein kinase inhibitor peptides 6-22-amide (100 nM) or 14-22-amide (5 µM) abrogated the effect. In line with that, Cx43 phosphorylation was reduced by PKA inhibitors and increased by activators, indicating that PKA mediated signaling mechanisms are involved in Cx43-mediated ATP release under these conditions.

Conclusion: The present study demonstrates that endothelial cells release ATP in a Cx43-dependent process during reoxygenation. Reoxygenation stimulated the cAMP/PKA signaling pathway which targets Cx43 at Ser 368 triggering the ATP release via Cx43. This reoxygenation-induced ATP release is enhanced by two chemically not

related connexin channel openers also targeting the cAMP/PKA pathway. Strengthening of this endogenous mechanism by direct activation of cAMP/PKA pathway and temporary opening of Cx43 hemichannels could represent a new therapeutic strategy to prevent acute endothelial barrier failure during reperfusion-injury.

# 7 ZUSAMMENFASSUNG

Reperfusionschäden stören die endotheliale Schrankenfunktion und können zur Entstehung von Ödemen führen. Hierdurch wird die Regeneration des reperfundierten Herzen beeinträchtigt. Wie kürzlich gezeigt wurde, schützt ATP, das spontan von Endothelzellen (EC) während der Reperfusion abgegeben wird, die endotheliale Schranke vor Reperfusionsschäden. Das Ziel der vorliegenden Studie war es, den Mechanismus der spontanen ATP-Freisetzung in Endothelzellen zu untersuchen. Zentrale Hypothese war hierbei, dass Connexin 43 (Cx43) an der endothelialen ATP-Freisetzung während der Reoxygenierung beteiligt sind. Es wurde beobachtet, dass die ATP Freisetzung in makrovaskulären EC des Schweines zu Beginn der Reperfusion Cx43-abhängig ist. Messungen der LDH-Aktivität ergaben, dass dieser Anstieg der ATP-Konzentration nicht auf Zelllyse zurückzuführen ist. Um den Mechanismus der ATP-Freisetzung zu untersuchen, wurden EC einerseits mit den selektiven Pannexin- und Connexininhibitoren wie Mefloquine (10µM) und Lanthan-(III)-chlorid (200µM), sowie andererseits mit den panspezifischen Inhibitoren wie 18α-Glycyrrhetinic acid (50 µM) oder Flufenamic acid (50 µM) vorinkubiert. Dies führte zu einer Reduktion der durch Reperfusion verursachten ATP-Freisetzung. Wurde dagegen Pannexin mittels Probenecid in einer Konzentration zwischen 1µM und 10µM, oder Mefloquine in einer Konzentration kleiner als 10µM unter sonst gleichen Bedingungen gehemmt, führte dies nicht zu einer Reduktion der ATP-Freisetzung. Dies ist ein Hinweis darauf, dass die ATP-Freisetzung eher durch Connexin als durch Pannexin erfolgt.

Diese Ergebnisse konnten durch eine spezifische Cx43-siRNA-Transfektionsstrategie bestätigt werden. Der Zusatz des anti-arrythmischem Peptids 10 (AAP10; 10µM) oder Alendronat (10µM), die Connexinkanäle öffnen, führte zu einer signifikanten Steigerung der ATP-Freisetzung im Vergleich zur reoxygenierten Kontrolle. Die durch Reoxygenierung induzierte ATP-Freisetzung wurde ebenfalls verstärkt, wenn spezifische Aktivatoren der PKA, wie 8-BrcAMP (1 µM), Sp8CPT (10 µM) und 6Bnz (10 µM) zu Beginn der Reperfusion zugesetzt wurden. Dagegen trat dieser Effekt durch die Gabe von H-89 (20 µM), bzw. KT-5720 (100 nM) sowie das PKA-inhibierende Peptid 6-22-amide (100 nM) oder 14-22-amide (5 µM) nicht auf. Gleichzeitig dazu bewirken PKA-

Inhibitoren eine Abnahme der Connexin43-Phosphorylierung, während PKA-Aktivatoren diese verstärken, Das deutet darauf hin, dass PKA-vermittelte Signaltransduktionsmechanismen an der Cx43-vermittelten ATP-Freisetzung beteiligt sind.

Schlussfolgerung: Die Ergebnisse der vorliegenden Studie zeigen, dass Schweineendothelzellen während der Reoxygenierung ATP mittels Cx43 freisetzen. Die Reoxygenierung stimuliert auch den cAMP/PKA-Signalweg, wobei der Angriffspunkt der Phosphorylierung das Serin an Position 368 von Connexin 43 ist. Durch Veränderungen an dieser Stelle wird die ATP-Freisetzung gesteuert. Die durch Reoxygenation verursachte ATP-Freisetzung wird durch zwei chemisch nicht verwandte Connexin-Kanalöffner verstärkt, welche beide auf den cAMP/PKA-Signalweg einwirken. Eine Verstärkung dieses endogenen Mechanismus durch direkte Aktivierung des cAMP/PKA-Signalweges und damit eine zeitlich begrenzte Öffnung des Connexin-Kanals könnte eine neue therapeutische Strategie sein, die akute endotheliale Schrankenstörung während der Reperfusion zu verhindern.

# 8 DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

---

(Kiran Khawaja)

# 9 APPENDIX

## 9.1 Chemicals and consumables

| | |
|---|---|
| 6-amino hexanoic acid | Merck, Darmstadt, Germany |
| Acrylamide solution (40%; wt/vol) | Amersham Pharmacia, UK |
| Acrylamide | Carl Roth, Karlsruhe, Germany |
| Ammonium persulfate | SERVA, Heidelberg, Germany |
| Ammonium sulfate | Merck, Darmstadt, Germany |
| ATP | Roche, Mannheim, Germany |
| Benzonase® | Merck, Darmstadt, Germany |
| Bisacrylamide solution (2%; wt/vol) | Amersham Pharmacia, UK |
| Bisacrylamide | Carl Roth, Karlsruhe, Germany |
| Bovine serum albumin | Sigma-Aldrich, Steinheim, Germany |
| Bromophenol blue | Sigma-Aldrich, Steinheim, Germany |
| Calcium chloride | Merck, Darmstadt, Germany |
| Culture dishes | BD, Heidelberg, Germany |
| Diethyl ether | Merck, Darmstadt, Germany |
| Dimethyl sulfoxide | Sigma-Aldrich, Steinheim, Germany |
| Di-sodium hydrogen phosphate | Carl Roth, Karlsruhe, Germany |
| Dithiothreitol | Amersham Pharmacia, UK |
| EDTA | Carl Roth Karlsruhe, Germany |
| EGTA | Boehringer, Mannheim |
| Endothelial cell basal medium® kit | PromoCell, Heidelberg, Germany |
| Endothelial cell growth supplement | PromoCell, Heidelberg, Germany |
| Eppendorf tubes (0.5, 1.5, 2 ml) | Eppendorf, Hamburg, Germany |
| Falcon tubes (50 ml, 12 ml) | BD, Heidelberg, Germany |
| FCS | PAA, Pasching, Austria |
| Filter papers | Biotech-Fischer, Reiskirchen, Germany |
| Glass cover slips | Menzel, Braunschweig, Germany |
| Glycerol (100%) | Sigma-Aldrich, Steinheim, Germany |
| Glycerol (87%) | Amersham Pharmacia, UK |

| | |
|---|---|
| Glycine | Carl Roth, Karlsruhe, Germany |
| HBSS | PAA, Pasching, Austria |
| hEGF | PromoCell, Heidelberg, Germany |
| HEPES | Sigma-Aldrich, Steinheim, Germany |
| LDH release Kit | Cyto-Tox One, Promega, Mannheim, Germany |
| Magnesium chloride | Fluka, Switzerland |
| Magnesium sulfate | Merck, Darmstadt, Germany |
| Manganese chloride | Merck, Darmstadt, Germany |
| ®-mercaptoethanol | Merck, Darmstadt, Germany |
| Methanol | Merck, Darmstadt, Germany |
| Millipore water | Millipore, Eschborn, Germany |
| Molecular weight marker | Sigma-Aldrich, Steinheim, Germany |
| Nitrocellulose membrane | Schleicher und Schuell, Dassel, Germany |
| Nonidet P-40 | Sigma-Aldrich, Steinheim, Germany |
| Penicillin/streptomycin | Gibco BRL, Eggenstein, Germany |
| Phosphorylase-b | Sigma-Aldrich, Steinheim, Germany |
| Phosphorylase-kinase | Sigma-Aldrich, Steinheim, Germany |
| Pipette tips | Eppendorf, Hamburg, Germany |
| Pipettes | Eppendorf, Hamburg, Germany |
| PKI | Calbiochem, Bad Soden, Germany |
| PMSF | Sigma-Aldrich, Steinheim, Germany |
| Ponceau S solution | SERVA, Heidelberg, Germany |
| Potassium chloride | Merck, Darmstadt, Germany |
| Potassium dihydrogen phosphate | Merck, Darmstadt, Germany |
| Rubber policeman | BD, Heidelberg, Germany |
| Scalpal (disposble) | Feather, Japan |
| See Blue® (pre-stained marker) | Invitrogen GmbH, Karlsruhe, Germany |
| Sodium azide | Merck, Darmstadt, Germany |
| Sodium bicarbonate | Carl Roth, Karlsruhe, Germany |
| Sodium chloride | Carl Roth, Karlsruhe, Germany |
| Sodium di-hydrogen phosphate | Carl Roth, Karlsruhe, Germany |

| | |
|---|---|
| Sodium dodecyl sulfate | SERVA, Heidelberg, Germany |
| Sodium fluoride | Sigma-Aldrich, Steinheim, Germany |
| Sodium hydroxide | Carl Roth, Karlsruhe, Germany |
| Sodium orthovanadate | Sigma-Aldrich, Steinheim, Germany |
| Sodium thioglycolate | Sigma-Aldrich, Steinheim, Germany |
| Sterile filters (0.22 µm) | Sartorius, Goettingen, Germany |
| Sterile pipettes | BD, Heidelberg, Germany |
| Sucrose | Sigma-Aldrich, Steinheim, Germany |
| Super signal-west® (ECL solution) | Pierce biotech, Bonn, Germany |
| Syringes (20 ml, 2 ml) | BD, Heidelberg, Germany |
| TEMED | Sigma-Aldrich, Steinheim, Germany |
| Transwell® membrane filters | Corning, NY, USA |
| Tricholoroacetic acid | Merck, Darmstadt, Germany |
| Tris base | Carl Roth, Karlsruhe, Germany |
| Tritone X-100 | SERVA, Heidelberg, Germany |
| Trypsin-EDTA | Gibco-BRL, Eggenstein, Germany |
| Tween 20 | Amersham Pharmacia, UK |
| Whatman® 3 MM filter paper | Millipore, Eschborn, Germany |

## 9.2 Pharmacalogical inhibitors and activators

| | |
|---|---|
| 4-22 amide | Biolog, Bremen, Germany |
| 16-22 amide | Biolog, Bremen, Germany |
| 18-α Glycyrrhetinic acid | Sigma-Aldrich, Taufkirchen, Germany |
| 8-Br-cAMP | Biolog, Bremen, Germany |
| AAP10 | Biotrend, Köln, Germany |
| Alendronate | Merck Bioscience, BadSoden, Germany |
| Flufenamic acid | Sigma-Aldrich, Taufkirchen, Germany |
| H-89 | Biolog, Bremen, Germany |
| KT5720 | Merck Bioscience, BadSoden, Germany |
| Lanthanum chloride | Sigma-Aldrich, Taufkirchen, Germany |
| Mefloquine | Sigma-Aldrich, Taufkirchen, Germany |

| | |
|---|---|
| Probenecid | Sigma-Aldrich, Taufkirchen, Germany |
| Sp-8-CPT | Biolog, Bremen, Germany |

## 9.3 Antibodies

*Primary antibodies*

| | |
|---|---|
| Anti Cx43 (Mouse IgG, polyclonal) | BD Bioscience, Heidelberg, Germany |
| Anti PhosphoCx43 Ser368 (Rabbit IgG, polyclonal) | Cell Signaling Technology, USA |
| Anti Cx37 (Mouse IgG, polyclonal) | Biotrend, Köln, Germany |
| Anti Cx40 (Rabbit IgG, polyclonal) | Biotrend, Köln, Germany |
| Anti Tubulin (Rabbit IgG, monolclonal) | Merck Bioscience, BadSoden, Germany |
| Anti Vinculin (Clone hVIN-1, mouse IgG, | Sigma-Aldrich, Taufkirchen, Germany |

*Secondary antibodies*

| | |
|---|---|
| Anti-mouse IgG HRP-conjugated | Amersham, Freiburg |
| Anti-rabbit IgG HRP-conjugated | Amersham, Freiburg |
| Anti-rabbit IgG Alexa 633-conjugated | Invitrogen, Karlsruhe |
| Anti-mouse IgG Alexa 488-conjugated | Invitrogen, Karlsruhe |

## 9.4 siRNA transfection

| | |
|---|---|
| Cx43 siRNA | Eurogentec, Köln, Germany |
| Control siRNA | Eurogentec, Köln, Germany |
| JetSI Endo | Peqlab Biotechnology, Erlangen, Germany |

## 9.5 Laboratory instruments

| | |
|---|---|
| ATP Luminometer | Turner Designs Instruments, Sunnyvale, CA |
| Beckman Allegra 64R centrifuge | Beckman Coulter, USA |

| | |
|---|---|
| Blotting chambers | Biotech-Fischer, Reiskirchen, Germany |
| Electrophoresis apparatus | Biometra, Goettingen, Germany |
| Gel documentation system | Quantity One series, Bio-Rad, Munich, Germany |
| Glass ware | Schott, Mainz, Germany |
| Hamilton syringe | Hamilton, Bonaduz, Switzerland |
| Incubators | Heraeus, Hanau, Germany |
| Laminar flow hood | Heraeus, Hanau, Germany |
| Tri-Carb 1600 TR liquid scintillation counter | Packard Instrument Company, CT, USA |
| Magnet stirrer | Jahnke und Kunkel, Staufen, Germany |
| Magnetic rack | DYNAL, Oslo, Norway |
| Neubauer chamber | Superior, Marienfeld, Germany |
| Phase contrast microscope | Olympus, Japan |
| pH-Meter | WTW-Weinheim, Germany |
| Photometer | Carl Zeiss, Jena, Germany |
| Power supply | Biometra, Goettingen, Germany |
| Rocker | Biometra, Goettingen, Germany |
| Table top (centrifuge) | Eppendorf, Hamburg, Germany |
| Vortexer | Heidolph, Kelheim, Germany |
| Water bath | Julabo, Seelbach, Germany |
| Water demineralisation unit | Millipore, Eschborn, Germany |

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*Kiran Khawaja*

**Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.**

**The curriculum vitae was removed from the electronic version of the paper.**

## ABSTRACTS

### Posters

**Khawaja, K**., Härtel, F.V., Gündüz, D., Reis, A., Nazli, S., Piper, H.M., and Noll, T (2010). Endothelial ATP release during reoxygenation is modulated by PKA targeting connexin 43. Joint Meeting of the Scandinavian and German Physiological Societies 27-30 March, Copenhagen, Denmark.

**Khawaja, K**., Härtel, F.V., Gündüz, D., Reis, A., Nazli, S., Piper, H.M., and Noll, T (2010). Endothelial ATP release during reoxygenation is modulated by PKA targeting connexin 43. ECCPS Retreat, Max-Planck Institute, Bad Nauheim, Germany

Haertel, F.V., Guenduez, D., **Khawaja, K.**, Weyand, M., Piper, H.M., Noll, T. (2009) Connexin 43-dependent ATP release protects endothelial cells from severe reperfusion-induced barrier failure. **Eur Heart J;** 30 (suppl) 644 (Annual Meeting of The European Society of Cardiaology, September 2009, Barcelona, Spain)

Haertel, F.V., Guenduez, D., **Khawaja, K.**, Weyand, M., Piper, H.M., Noll, T. (2009) Connexin 43-mediated ATP release reduces reperfusion-induced barrier failure in endothelial cells. ECCPS Symposium, Bad Nauheim, Germany

Haertel, F.V., Guenduez, D., **Khawaja, K.**, Weyand, M., Piper, H.M., Noll, T. (2008) Connexin 43-Mediated ATP Release Reduces Reperfusion-Induced Barrier Failure in Endothelial Cells, **Circulation**;118:S_575 (American Heart Association, Scientific Session, Nov. 2008, New Orleans, LA USA)

Haertel, F.V., Guenduez, D., **Khawaja, K.**, Urban, D., Weyand, M., Piper, H.M., Noll, T. (2008) Freisetzung von ATP aus Endothelzellen via Connexin 43 schützt vor dem reperfusions-induzierten Schrankenversagen. 74$^{th}$ Annual Meeting of The German Society of Cardiology (DGK), Mannheim, Germany.

**Khawaja, K.**, Weyand, M., Härtel, F.V., Noll, T.,Piper, H.M. Role of Cx43 in ATP mediated protection of endothelial barrier failure during hypoxia/reperfusion. 1st conference organized by Giessen Graduate School for the Life Sciences.2008. Giessen, Germany

## Oral Presentations

**Khawaja, K**., Härtel, F.V., Gündüz, D., Reis, A., Nazli, S., Piper, H.M., and Noll, T (2010). Endothelial ATP release during reoxygenation is modulated by PKA targeting Cx43. 76th Annual Meeting of The German Society of Cardiology (DGK), Mannheim, Germany.

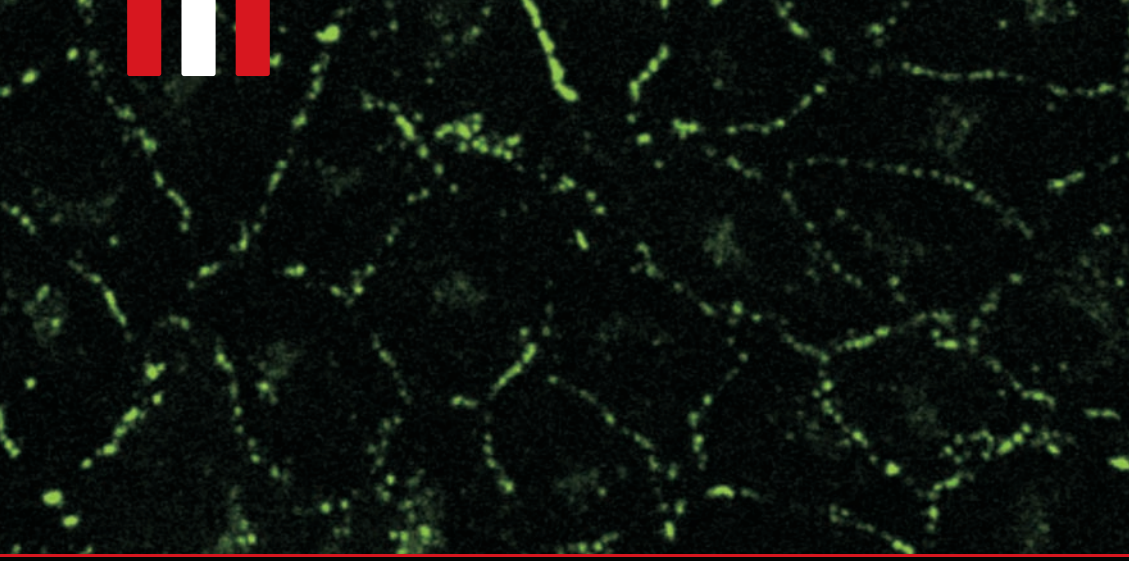
Endothelial ATP release during reoxygenation is modulated by PKA targeting Connexin 43 hemichannels. 2nd conference organized by Giessen Graduate School for the Life Sciences, 2009, Giessen, Germany.

Härtel, F.V., **Khawaja, K.**, Gündüz, D., Piper, H.M., Noll, T. (2008). Role of Connexins in ischemia-reperfusion-induced injury of endothelial cells. 52nd GTH Congress, Gesellschaft für Thrombose- und Haemostaseforschung e.V., Wiesbaden, Germany.

## Awards:

Best oral presentation:

Endothelial ATP release during reoxygenation is modulated by PKA targeting Connexin 43 hemichannels. 2nd conference organized by Giessen Graduate School for the Life Sciences, 2009, Giessen, Germany.



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