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Nitric oxide enhances de novo formation of endothelial gap junctions

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Abstract

Objective: Gap junctions (formed by connexins, Cx) are important for functional coordination of cells in the vascular wall. However, little is known about their physiological regulation in this tissue. We examined the effects of nitric oxide (NO), an important mediator of vasomotion, wound healing and angiogenesis, on the formation of gap junctions in endothelial cells (human umbilical vein endothelial cells, HUVEC). **Methods:** Flow cytometry was used to determine dye transfer through newly formed gap junctions between acutely coincubated HUVECs. Parallel experiments in wild-type HeLa cells (no connexins) and transfected HeLa cells exclusively expressing Cx43, Cx40 or Cx37 were performed to determine the specific role of Cx subtypes. The intracellular distribution of Cx40 was examined after fractionation with triton by Western blotting. Intracellular levels of cGMP and cAMP were measured by radioimmunoassay. **Results:** The NO donor SNAP (1 μM) enhanced gap-junctional coupling in HUVECs by about 40%. This was associated with an enhanced incorporation of Cx40 into the membrane. Both effects were restricted to Cx40 as analyzed in experiments with Cx-selective HeLa cells. The NO-induced increase in cell coupling was elicited by a corresponding rise of cGMP, which secondarily increased intracellular cAMP levels. The latter was an integral part of the signal cascade, since the protein kinase A (PKA) inhibitor H89 blocked the SNAP-induced incorporation of Cx40 into the plasma membrane. **Conclusions:** We conclude that NO is a potent modulator of gap-junctional coupling in endothelial cells. It enhances de novo formation of endothelial gap junctions by increasing incorporation of Cx40 into the plasma membrane due to PKA activation.

Keywords: Cell communication; Endothelial function; Gap junctions; Nitric oxide; Protein kinases

1. Introduction

Transmission of signals from one cell to another through the exchange of ions, metabolites or second messengers (e.g. cAMP or cGMP) maintains cellular and tissue homeostasis. Therefore, in most tissues, the cytoplasms of individual cells are metabolically and/or electrically coupled to that of neighboring cells through gap junctions [1]. The basic subunits of gap junctions are connexins (Cx), forming hexameric hemichannels (connexons). A gap junction is formed by two hemichannels from adjacent cells [2]. Gap junctions are non-specific channels exhibiting low electrical resistance and allowing passage of small molecules with a molecular mass of less than 1.5 kDa [1]. Alterations in this cellular communication system could play a role in modulating the response to certain stimuli and in coordinating cellular actions, e.g. during differentiation and remodeling: sprouting of endothelial cells requires communication through gap junctions [3] and gap-junctional communication is consistently enhanced in migrating endothelial cells [4]. Endothelial cells express mainly Cx43, 40 and 37 [5], which allows a variety of homo- and heterotypic channels to be formed [6].

Regulation of gap-junctional communication in response to physiological stimuli occurs at different levels. Changes in gap-junctional coupling may be mediated by alterations of single channel electrical conductances or the open probability of gap-junction channels [7]. Alternatively, cell coupling may be influenced by the balance between the incorporation of new Cx into the plasma membrane and their degradation, a very potent mechanism as evidenced by the unusually short half-life of 1–3 h of Cx in the membrane [8,9]. In this context, phosphorylation of connexins might play a role as their intracellular C-termini contain phosphorylation sites for a variety of protein kinases [10]. Mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and v-Src kinase are able to phosphorylate Cx43 [11]. Similarly, the C terminus of

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Cx40 contains phosphorylation sites for PKC-, cGMP- or cAMP-dependent protein kinase [12]. Changes in the phosphorylation status of Cx have been shown to mediate alterations in cell coupling and gap junctional single conductance state [13,14].

Several studies also show the importance of gap-junctional coupling not only between endothelial cells, but also between endothelium and smooth muscle. The vasodilatory effect of the endothelium-derived hyperpolarizing factor (EDHF) seems to be dependent on gap junctions between endothelial and smooth muscle cells [15,16]. Cellular communication through gap junctions is also required for the propagation of vasomotor signals along the vessels [17]. Under in vivo conditions, an attenuation of a conducted

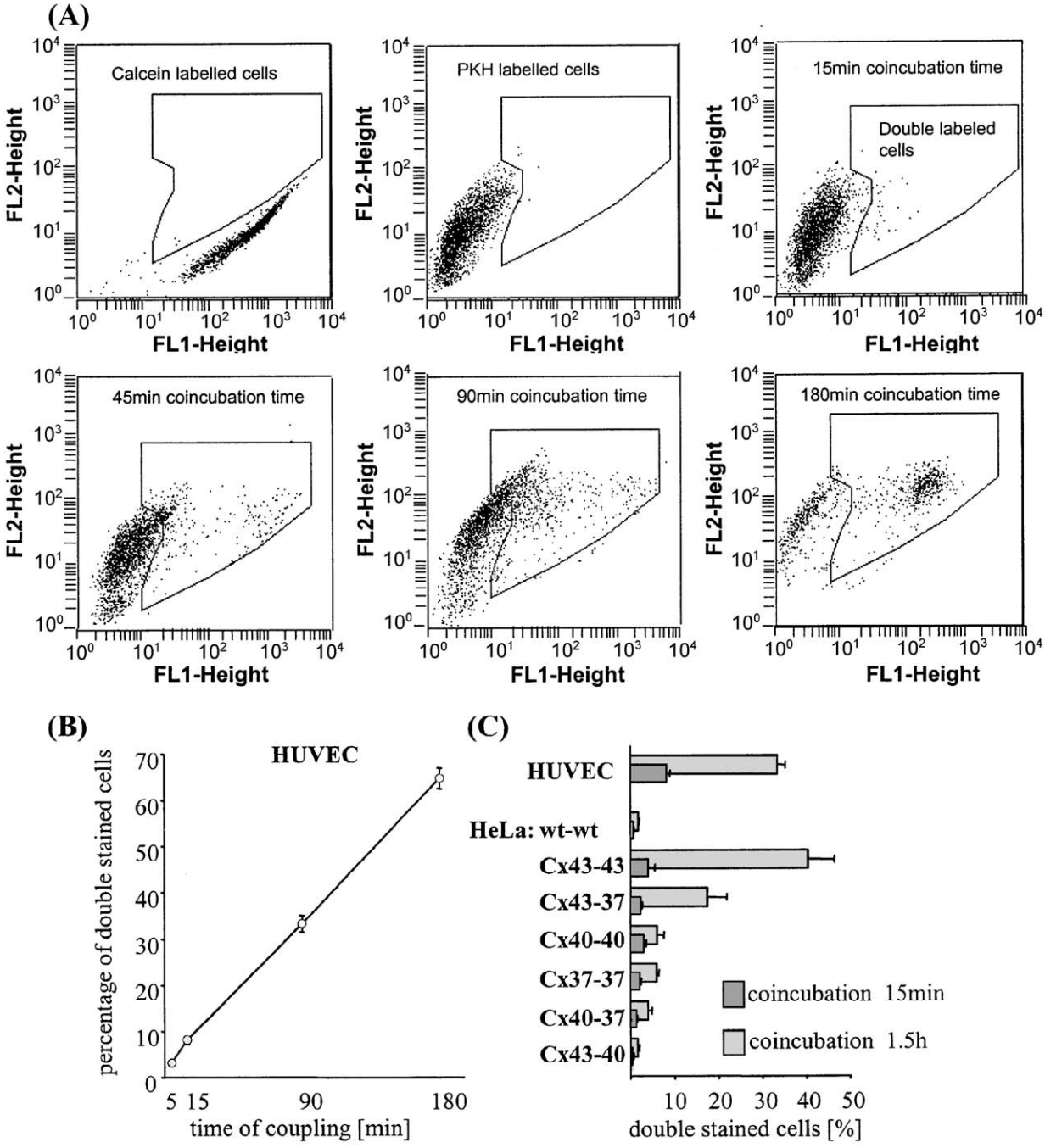

Fig. 1. Cell coupling in HUVEC- and Cx-transfected HeLa cells. (A) Flow cytometry diagrams showing two differently labelled cell populations and in the following the increase in double-stained cells over coincubation time. FL-1: calcein fluorescence, FL-2: PKH fluorescence. (B) The percentage of double-stained HUVEC increased linearly over time ($n = 35$). (C) The percentage of double-stained cells showed an increase over time in Cx-expressing cells. HeLa-wt cells not expressing connexins showed no detectable dye transfer. In the other experimental groups (with the exception Cx43–Cx40), dye transfer was statistically significant ($p < 0.05$). The percentage of double-stained cells depended on the kind of Cx present in the cells ($n = 35$).

dilation caused by acetylcholine was found in Cx40-deficient mice [18], further emphasizing the role of Cx in regulating local vascular tone. Though the role of nitric oxide (NO) in the regulation of gap junctional coupling has been examined in some cell types [19,20], so far, little is known about the influence of this vasoactive factor on coupling between vascular cells. This mediator is of special interest because it is involved not only in vasodilatation, but also in cell migration, proliferation, inflammation and angiogenesis, where de novo formation of gap junctions might be especially important. Therefore, we investigated whether and by which cellular mechanisms NO affects gap-junctional communication of endothelial cells (human umbilical vein endothelial cells, HUVEC) in a coincubation model, which favours de novo formation of gap junctions. We further investigated whether potential effects of NO on endothelial cell coupling could be attributed to effects on one single type of connexin or were due to a general effect on all of them.

2. Methods

2.1. Cell culture

Endothelial cells were isolated from freshly obtained human umbilical cord veins (HUVEC) according to Jaffe et

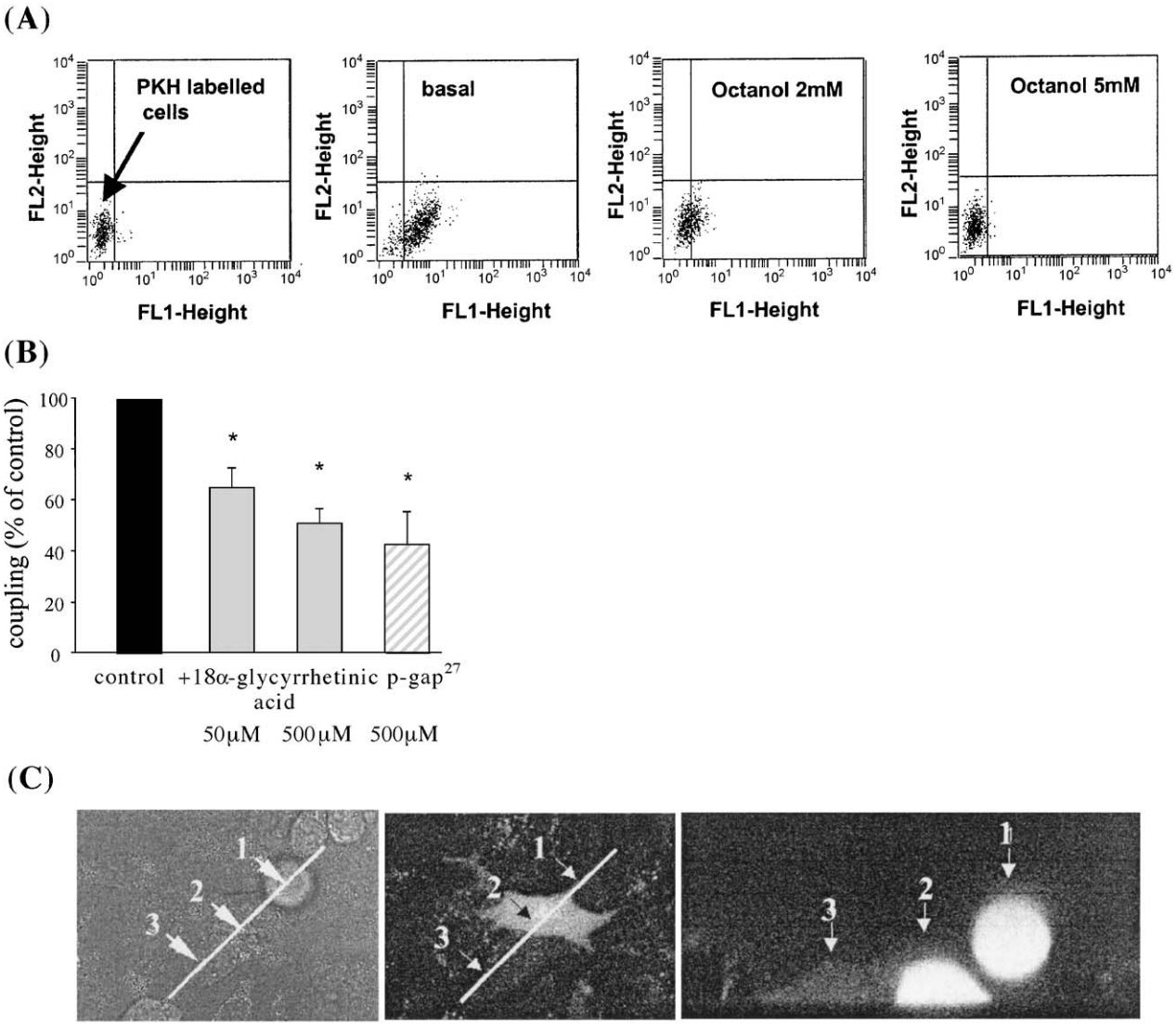

Fig. 2. Reduction of dye transfer by different inhibitors of gap-junctional coupling in HUVEC. (A) Flow cytometry diagrams of HUVEC depict that the gap junction inhibitor octanol inhibited dye transfer concentration dependently with complete inhibition at 5mM. FL-1: calcein fluorescence, FL-2: PKH fluorescence. (B) Statistical analysis displaying that the gap-junctional inhibitors 18α-glycyrrhetinic acid and *p*-gap27 inhibited dye transfer by about 50–60% ($n = 5–7$). *$p < 0.05$ vs. control. (C) Confocal laser scanning micrograph of calcein spreading between HUVECs. Calcein-labelled HUVECs were placed on top of an unlabelled HUVEC monolayer. Left: phase contrast, middle: calcein fluorescence, right: z-sectioning. Arrow 1: donor cell; arrow 2: recipient cell, 1st generation; arrow 3: recipient cell, 2nd generation. The white line indicates the position where the z-scan was performed.

al. [21]. Briefly, veins were filled with phosphate-buffered saline containing 2.4 U/ml dispase (Roche, Mannheim, Germany). After 30 min, incubation-detached cells were harvested and seeded into cell culture flasks using endothelial cell growth medium (Promocell, Heidelberg, Germany). After reaching confluence, HUVECs were sub-cultivated in a humidified atmosphere at 37 °C and 5% CO_2. In all experiments, only cells of second and third passage were used.

For studying effects of individual types of connexins, human cervical carcinoma cells (HeLa, ECACC No. 96112022) transfected with expression vectors containing either one of the coding sequences for murine connexin 43, 40 or 37 were used [22] with wild-type HeLa cells as controls. These cells were a generous gift from Prof. Willecke, Bonn. HeLa cells were cultivated in DMEM, 10% FCS, 2.5 mM glutamine and antibiotics (penicillin and streptomycin). For connexin transfected HeLa cells, the medium was supplemented with 1 μg/ml puromycin (Sigma, Deisenhofen, Germany).

Confluent HeLa or HUVEC monolayers were studied under control conditions or after incubation with one or a combination of the following compounds:

N^{ω}-nitro-L-arginine (L-NA, 30 μM, 45 min)
superoxide dismutase (SOD, 200 U/ml, 30 min)
1-*H*-[1,2,4]-oxadiazole-[4,3]-quinoxalin-1-one (ODQ, 10 μM, 45 min)
8-Br-cGMP (100 μM, 30 min)
Brefeldin A (BFA, 2 mM, 30 min)
18α-glycyrrhetinic acid (50/500 μM, 20 min)
p-gap27 (SRPTEKTVFTV-OH, 500 μM, 20 min)
forskolin (FSK, 100 μM, 20 min)
N-[2-*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl (H89, 1 μM, 30 min)
S-nitroso-*N*-acetyl-DL-penicillamine (SNAP, 1 μM, 15 min).

Sham-treated cells from the same culture batch served as corresponding controls for paired studies.

2.2. Quantification of cell communication

To determine cell coupling, a flow cytometry based method according to Tomasetto et al. [23] was used. In brief, populations of confluent HUVEC or HeLa monolayers were labeled with calcein-acetoxymethyl ester (0.1 μM, 10 min, Molecular Probes, Eugene, OR, USA) in 24-well dishes. This form of dye is membrane permeable and is converted to an impermeable one after cleavage by intracellular esterases. Prior to incubations with different agents as indicated above, remnants of calcein dye were removed by triplicate washings in PBS.

A second cell population (dye acceptor cells) that had been labeled with the membrane-bound dye PKH26 (5 μM, 5 min, Sigma) was trypsinized and added to the calcein-labeled donor cells. Coincubation was initiated by centrifugation (15 × *g* for 3 min) and lasted for various periods of time to allow for dye transfer. Subsequently, the only loosely attached acceptor cells were removed by washing and analyzed by flow cytometry (>10,000 acceptor cells) for double staining, indicating transfer of the calcein dye into the PKH-labeled cells. The fluorescence was measured (FACScan, Becton-Dickinson, Heidelberg, Germany) at an excitation wavelength of 488 nm. Emission light of calcein and PKH was determined at 530 (FL-1) and 575 nm (FL-2), respectively. Dot plots (FL-1/FL-2) were analyzed using Cell quest 3.1™ software. Dye coupling was determined as percentage of double stained cells.

2.3. Western blotting

Confluent HUVEC or HeLa cells were collected in lysis buffer (20 mM KH_2PO_4 pH 7.0, 1 mM EDTA, 1 mM Pefa block®, 10 μg/ml aprotinin, 0.5 mg/ml leupeptin, 0.7 μg/ml pepstatin, 50 mM NaF, 40 mM Na-pyrophosphate and 1 mM Na_3VO_3) and kept at 4 °C overnight. Triton X-100 was added to a final concentration of 1% and the samples were incubated further on ice for 30 min. For crude membrane preparation, two thirds of the lysate was centrifuged for 2.5 h (10,000 × *g*, 4 °C), whereas one third was saved as ''total cell lysate''. The

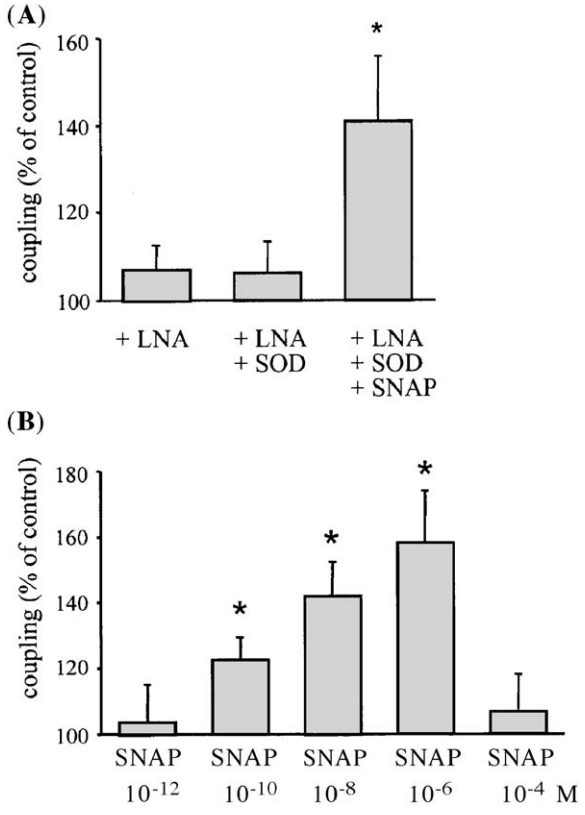

Fig. 3. Influence of NO on cell coupling. (A) Preincubation of HUVEC with L-NA and SOD had no effect on gap-junctional coupling, but addition of the NO donor SNAP enhanced cell coupling ($n = 25–31$). (B) SNAP increased gap-junctional coupling in a concentration-dependent manner ($n = 7–11$). *$p < 0.05$ vs. untreated control.

Table 1
NO exerted no effect on cell coupling mediated by Cx43 or Cx37

	Cx43–43 (%)	Cx43–37 (%)	Cx37–37 (%)
L-NA	12.4 ± 9.6	18.3 ± 14.1	10.7 ± 9.6
L-NA + SOD	18.3 ± 10.4	10.8 ± 18.6	18.4 ± 13.9
L-NA + SOD + SNAP	18.4 ± 16.4	16.9 ± 14.2	14.9 ± 12.2

HeLa cells transfected with Cx43 or Cx37 showed no NO-induced increase in dye transfer. Values are changes in cell coupling (% of control, $n = 15–20$). All data are not statistically different from the respective controls.

supernatant of the centrifuged lysates (''triton soluble fraction'') was separated from the pellet (''triton insoluble fraction''). Preparations were separated by SDS-PAGE (10%) and transferred onto a nitrocellulose membrane (Optitran, Schleicher and Schuell, Dassel, Germany) using a semi-dry blotting device (1 h, 0.7 mA/cm^2). After blocking, the membrane was probed overnight at 4 °C with specific antibodies to either Cx43 (Transduction laboratories, Lexington, KY), Cx40 or Cx37 (Biotrend, Cologne, Germany). Specific bands were identified using horseradish-conjugated 2nd antibodies (Sigma) and chemi-luminescence reaction with luminol and cumaric acid as substrates (Sigma).

2.4. Measurement of intracellular cGMP and CAMP

Cyclic nucleotide concentrations were determined with the ^{125}I proximity assay (SPA) system (Amersham Pharmacia Biotech, Freiburg, Germany). In case of cGMP measurement, the culture medium was replaced by 100 μl Lockes solution pH 7.4 (154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 3.6 mM $NaHCO_3$, 5.6 mM dextrose) supplemented with the phosphodiesterase inhibitor Zaprinast (30 μM). HUVECs were incubated with either SNAP or 8-Br-cGMP. To determine intracellular cGMP, the Lockes solution was replaced by ice cold 50 mM NaAc (pH 4.0). For determination of total cAMP levels (intracellular and cell supernatant cAMP), Lockes solution was not removed. Finally, cells and their supernatants were lysed by snap freezing in liquid nitrogen and used for the assay as described in the manufacturer's manual.

2.5. Materials

Unless indicated otherwise, all chemicals were of analytical grade and were purchased from Sigma. *p*-gap27 (SRPTEKTVFTV-OH), a peptide homologous to the extracellular gap27 sequence of Cx32 that has been identified as an effective gap junction blocker [24], was synthesized by BioSource (Solingen, Germany) and dissolved in PBS with 0.7 mM Ca^{2+} and 0.25 mM Mg^{2+}.

2.6. Confocal microscopy

To test whether dye transfer was specific and locally limited, confocal images were obtained of calcein-labelled HUVEC, coincubated with HUVEC that had been grown on glass cover slips. Dye transfer was visualized with a Zeiss LSM 410 confocal microscope and a 60 × oil immersion objective. Z-sections were obtained using the LSM 410 software.

2.7. Data analysis

All values are expressed as means ± S.E.M. Data were analyzed for statistical differences by the Student's *t*-test for paired data. Differences were considered significant when the error probability was $p \leq 0.05$ (treatment vs. time-matched control after testing normal distribution of the differences).

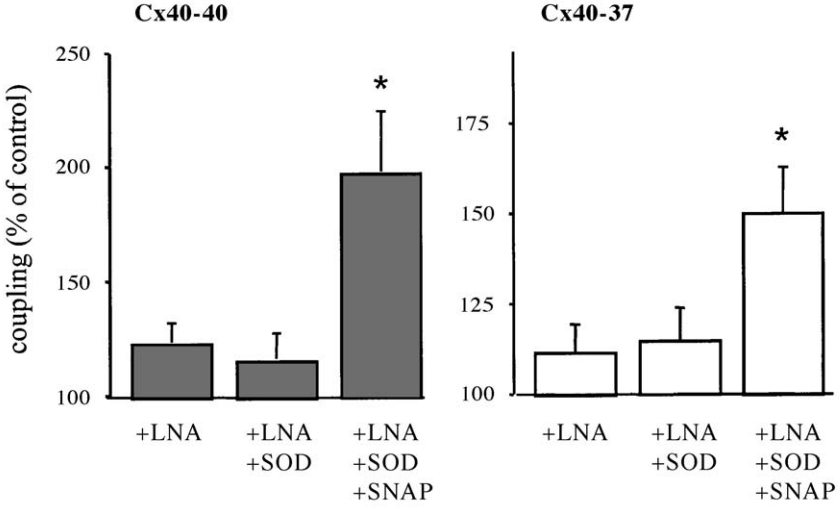

Fig. 4. Increase in cell coupling by NO is mediated via Cx40. NO enhanced gap-junctional coupling in HeLa cells transfected with Cx40 ($n = 20–25$). *$p < 0.05$ vs. control.

3. Results

3.1. Cell coupling of unstimulated HUVEC and HeLa cells

Cell coupling increased linearly with coincubation time. The percentage of double-stained HUVECs amounted to 3.1 ± 0.3%, 8.2 ± 0.8%, 33.3 ± 1.8% and 64.8 ± 2.3% after 5, 15, 90 and 180 min, respectively ($n = 35$, Fig. 1A,B). In Cx-deficient HeLa wild-type (HeLa-wt) cells, virtually no double-stained cells (< 2%) could be detected, even after 90 min of coincubation (Fig. 1C). This seems to be the "background" dye transfer in our model. HeLa cells transfected with either Cx43, 40 or 37 showed time-dependent dye transfer. The quantitative amount was dependent on the type of the Cx: coupling of Cx43 HeLa cells with Cx43, as well as coupling of Cx43 with Cx37 HeLa cells, showed the highest amount of double-stained cells, to an extent that was comparable to HUVECs. Dye transfer was significantly lower in homomeric as well as homomerical–heterotypical coupling of HeLa Cx40 and Cx37 cells (Fig. 1C). Cx40 and Cx43 showed virtually now coupling (< 2%), as has been described before in these HeLa cells [22]. The gap junction inhibitor octanol completely inhibited dye transfer (Fig. 2A). 18α-glycyrrhetinic acid, a different gap junction blocker, attenuated the percentage of double-stained HUVEC in a concentration-dependent manner by − 35.5 ± 7.7% (50 μM, $n = 7$) and − 49.3 ± 5.3% (500 μM, $n = 7$). A gap junction inhibitory peptide [24] also decreased dye transfer by − 57.6 ± 12.3% (500 μM, $n = 5$) as compared to untreated HUVEC (Fig. 2B).

The specificity and local limitation of dye transfer was visualized by confocal microscopy. Fig. 2C depicts a z-section through the calcein donor cell as well as through two recipient cells.

3.2. NO enhances gap-junctional coupling

To investigate the effect of NO on gap-junctional coupling independent of endogenous formation of NO by NO synthase or consumption of NO by endothelial superoxide, we pre-incubated HUVECs with L-NA, an inhibitor of NO synthase and the enzyme SOD. L-NA alone or in combination with SOD had no effect on dye transfer. In contrast, addition of the NO donor SNAP (1 μM) increased the percentage of double stained cells by + 40.9 ± 15.0% ($n = 31$, Fig. 3A). The SNAP-induced increase in dye coupling was concentration dependent over a wide range (10^{-10}–10^{-6} M; Fig. 3B). Interestingly, SNAP in a concentration of 10^{-4} M no longer increased cell coupling (Fig. 3B).

3.3. The NO-induced rise in cell coupling is mediated by Cx40

As HUVECs express Cx43, 40 and 37, we examined the effect of NO on specific types of connexin in HeLa cells transfected with either one of these Cx. HeLa cells transfected with Cx43 or Cx37 showed no increase in dye transfer when incubated with SNAP. The same was true for coupling of Cx43 with Cx37 (Table 1). In contrast, SNAP increased the percentage of double-stained cells in only Cx40-transfected HeLa cells by + 97.8 ± 26.4% ($n = 24$), and in HeLa Cx40 cells when coupled with Cx37 transfected HeLa cells (+ 50.2 ± 12.4%, $n = 24$, Fig. 4).

3.4. The increase in Cx40-dependent coupling is mediated by cyclic nucleotide-dependent mechanisms involving activation of PKA

cGMP measurements in HUVEC showed a SNAP-induced rise in cGMP (+ 81.3 ± 28.9%, $n = 10$), which could

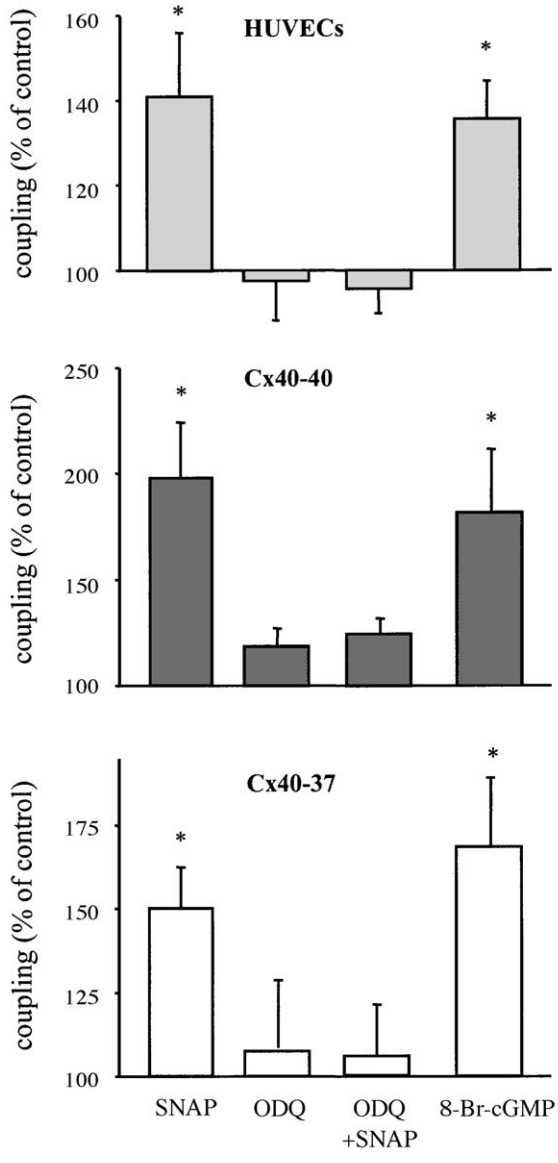

Fig. 5. The increase in Cx40 coupling depended on cGMP. In HUVECs and HeLa transfectants, the increase of coupling by SNAP was abolished by ODQ (inhibitor of guanyl cyclase). The membrane permeable analogue 8-Br-cGMP mimicked the effect of SNAP ($n = 20$–25). *$p < 0.05$ vs. SNAP.

be inhibited by ODQ, an inhibitor of the soluble guanylyl cyclase (− 14.1 ± 14.6%, $n = 8$). Preincubation of HUVECs as well as of Cx40-transfected HeLa cells with ODQ prevented the SNAP-induced increase in dye transfer (Fig. 5). Conversely, 8-Br-cGMP, a stable membrane permeant cGMP analogue, increased the percentage of double-stained cells to a similar extent as SNAP (Fig. 5), an effect which was pronounced most when only Cx40 was present. As we could not detect cGMP-dependent protein kinase in HUVEC lysates via western blot (data not shown), we investigated whether cGMP affected the cAMP level through its known effect on a cGMP-dependent phosphodiesterase (PDEIII). Treatment of HUVECs with SNAP or 8-Br-cGMP indeed increased cAMP levels by + 22.4 ± 3.3% ($n = 8$) or + 22.3 ± 6.9% ($n = 16$).

The cAMP-elevating compound forskolin (FSK) also enhanced the percentage of double-stained cells by + 102 ± 12.1% ($n = 66$, Fig. 6). This increase was reduced after preincubation with the protein kinase A (PKA) inhibitor H89 by about 50% ($n = 31$). The 8-Br-cGMP-induced increase in dye transfer was similarly reduced by H89 ($n = 32$, Fig. 6). Treatment of HUVECs with a combination of FSK and 8-Br-cGMP enhanced cell coupling synergistically (+ 181.2 ± 27.3%, $n = 25$, Fig. 6). H89 alone had no effect on coupling.

3.5. cAMP-induced PKA-dependent increase of translocation of Cx40 into the cell membrane

To distinguish whether NO increased the permeability for fluorescent dye of existing gap junctions or augmented incorporation of new Cx into the plasma membrane, HUVECs were incubated with Brefeldin A (BFA). BFA,

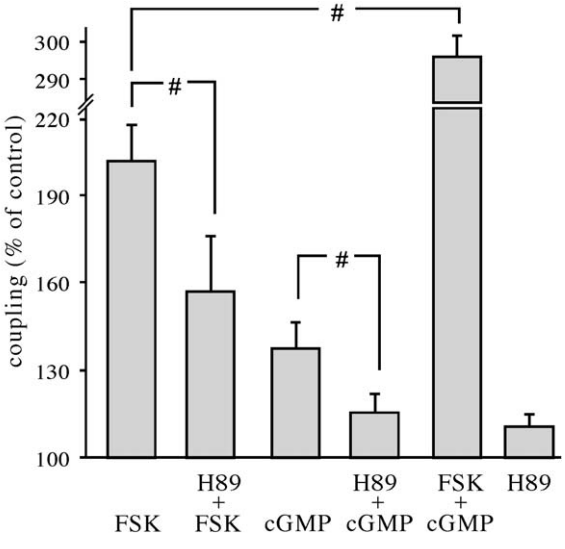

Fig. 6. The NO-induced increase in cell coupling is mediated by activation of PKA. Elevation of intracellular cAMP by forskolin (FSK) or 8-Br-cGMP (cGMP) enhanced coupling in HUVECs. This was PKA dependent, as both the FSK- and 8-Br-cGMP-induced increase in dye transfer was partially inhibited by the PKA inhibitor H89 ($n = 50–70$). #$p < 0.05$.

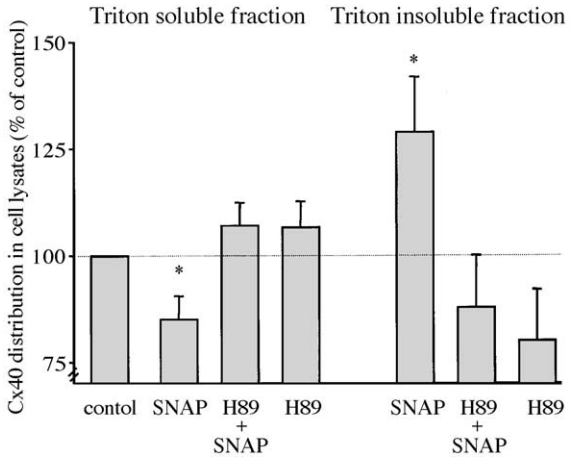

Fig. 7. NO-induced redistribution of Cx40 from the triton-soluble cell lysate to the triton-insoluble fraction. As detected by western blot, SNAP induced a PKA-mediated redistribution of Cx40 from the triton-soluble fraction to the triton-insoluble fraction which was inhibited by the PKA inhibitor H89 ($n = 15–16$). *$p < 0.05$ vs. untreated control.

which inhibits protein translocation from the Golgi apparatus to the plasma membrane, did not affect the percentage of double-stained cells as compared to controls (− 1.2 ± 9.1%, $n = 22$), but prevented the SNAP-induced increase in dye transfer (+ 2.0 ± 8.9%, $n = 22$). Furthermore, as determined by western blot, the Cx40 distribution in cell lysates was altered by SNAP: Cx40 in the triton-soluble fraction of the cell lysate decreased by − 14.8 ± 5.4% ($n = 16$), while it increased it in the triton-insoluble fraction by + 28.8 ± 12.9% ($n = 16$). This SNAP-induced redistribution of Cx40 was prevented by the PKA inhibitor H89 ($n = 14–16$, Fig. 7).

4. Discussion

This study demonstrates that NO enhances coupling between acutely coincubated endothelial cells (HUVEC). This is a Cx40-dependent phenomenon induced by a signal cascade involving the second messengers cGMP and cAMP, as well as protein kinase A. The most likely way by which this may occur is, according to our findings, by the de novo formation of gap junctions from preexisting Cx.

To determine gap-junctional coupling, we used a well-established flow cytometric method described by Tomasetto et al. [23] and others [25]. With this experimental setup, dye transfer is not determined within a confluent monolayer where cells are already coupled. Rather, de novo formation of gap junctions between two different cell populations is studied. Control experiments (no dye transfer between HeLa-wt, 50–100% inhibition of cell coupling with three different gap-junction inhibitors, direct visualization of dye distribution by confocal microscopy) confirmed that dye transfer was indeed dependent on gap junctions.

To determine the effect of NO on gap-junctional coupling, we used SNAP, a NO donor which significantly enhanced

gap-junctional coupling even in concentrations as low as 10^{-10} M. The maximum effect was observed at 1 μM SNAP, corresponding to about 2 nM NO [26]. Since agonists that cause liberation of endogenous NO from HUVECs (e.g. bradykinin) do so by increasing intracellular calcium levels and, thereby, induce a broad variety of signal cascades, we did not examine such drugs, but solely added NO exogenously. Though this is a limitation of our study, the broad range of concentrations of SNAP employed should fall within the nanomolar range of physiologically occurring NO levels.

A possible inactivation of endogenous or exogenous NO by superoxide anions which are produced under basal conditions in endothelial cells [27] was excluded by supplementing superoxide dismutase (SOD). Surprisingly, SNAP concentrations of 10^{-4} M did not increase cell coupling, suggesting an inhibitory effect of the highly reactive NO on a variety of signal molecules, as NO in higher concentrations is known to oxidize—SH or nitrosylate—OH groups of proteins [28]. Effects of NO can be sometimes be related to an indirect action of this small reactive molecule as a radical scavenger [29]. However, since SOD had no effect per se in our model, it is unlikely that the decrease of superoxide levels by NO played a role in the observed changes of cell coupling.

As HUVECs express three different types of connexins, Cx43, Cx40, and Cx37 [5], we analyzed whether the effects of NO on endothelial cell coupling could be specifically attributed to one single Cx. For these experiments, HUVECs were unsuitable as their various connexins could not be analyzed separately. Therefore, HeLa cells were used as additional experimental tools, as they do not express Cx in their wild-type form. Separate transfection with either Cx43, Cx40 or Cx37 [22] allowed us to study the effect of NO on only one type of connexin at a time in this model. NO had no effect on cell coupling in Cx43- or Cx37-transfected HeLa cells. In contrast, NO increased dye transfer when Cx40 was present, as not only coupling of HeLa cells transfected with Cx40 was augmented, but also gap-junctional coupling of Cx40 in combination with Cx37.

Enhanced coupling by NO in HUVECs as well as in Cx40-transfected HeLa cells was mediated by cGMP: cGMP levels rose after treatment with SNAP, preincubation with an inhibitor of the soluble guanylyl cyclase, ODQ, abolished the increase in NO-induced cell coupling and a stable cGMP analogue, 8-Br-cGMP, mimicked the NO effect. Usually, cGMP-dependent effects of NO are mediated by a cGMP-dependent kinase (PKG), e.g. in smooth muscle cells [30]. However, in agreement with previous investigations [30], we could not detect PKG in HUVEC cell lysates (data not shown), making a role for PKG in this context very unlikely. Since cGMP is known to directly affect the activity of cAMP-cleaving phosphodiesterases (PDE) II and III in many cell types [31], we investigated whether NO also affected intracellular cAMP levels. Since intracellular cAMP concentrations in HUVEC increased after NO or 8-Br-cGMP stimulation, and since the adenylyl cyclase activator forskolin (FSK) had an even higher effect than NO, it seems most likely that an increase of cAMP is mandatory for the rise in cell coupling due to treatment with SNAP. A cGMP-dependent inhibition of PDE III by the NO donor sodium nitroprusside was observed in the isolated perfused rat kidney, leading to increased cAMP levels [32]. The combination of 8-Br-cGMP with FSK enhanced endothelial cell coupling in a synergistic manner. Likewise, nitroprusside enhanced cAMP levels in platelets causing synergistic effects with an adenylyl cylcase activator on platelet function [33]. Synergistic effects of cAMP- and cGMP-elevating vasodilators were also found in the microcirculation in vivo [34]. The cAMP-dependent increase in cell coupling was presumably mediated by protein kinase A (PKA), as the PKA inhibitor H89 blocked both the increase in gap-junctional coupling due to forskolin as well as that induced by 8-Br-cGMP. The exact mode of action can, however, not be determined in our model, since in addition to indirect effects of cGMP on PKA via inhibition of phosphodiesterases, also direct activation of PKA by cGMP has been described [35,36]. Thus, the increase of cAMP we have seen after treatment with SNAP could only be an epiphenomenon. Whether the influence on gap-junctional coupling by NO or by mediators directly activating PKA is more prominent in vivo cannot be decided at the moment.

Though our experimental model was designed to analyze de novo formation of gap junctions, it cannot be fully excluded that NO might also modulate the conductivity of already existing gap junctions. Therefore, to determine whether PKA activation by NO increased incorporation of Cx into the plasma membrane, HUVECs were preincubated with Brefeldin A (BFA), which inhibits the translocation of proteins from the Golgi apparatus to the plasma membrane. The fact that BFA pretreatment blocked the SNAP-induced increase in gap-junctional coupling strongly suggests that NO indeed augmented cell coupling by an enhanced incorporation of new Cx into the plasma membrane. After prolonged BFA treatment (>90 min), gap-junctional coupling was reduced by $-28.5 \pm 2.9\%$ ($n = 10$, $p < 0.001$ vs. control; data not shown), reflecting the short half-life of about 1.5–3 h [9].

To further investigate the incorporation of Cx into the plasma membrane, we determined the distribution of Cx40 in cell lysates. Musil et al. have shown that functional Cx43 incorporated in the membrane is Triton X-100 insoluble, possibly due to strengthened anchorage to the membrane as a result of phosphorylation during the translocation process. In contrast, Cx43 still processed in the ER or Golgi apparatus is triton soluble [37]. NO decreased the amount of Cx40 in the triton-soluble fraction of the cell lysates and correspondingly increased it in the triton-insoluble fraction, indicating a shift of Cx40 from the cytoplasmic compartments to the plasma membrane under the assumption that Cx40 and Cx43 show similar biophysical properties. This redistribution was prevented by preincubation with the PKA inhibitor H89, suggesting that the incorporation of Cx into the plasma membrane required PKA activation. PKA mediated de novo formation of gap junctions from preexisting connexins could

be induced in several ways: the constant flux of Cx to the plasma membrane is mediated by a vesicular transport pathway [38,39], and PKA plays a role in the incorporation of vesicles into the plasma membrane and facilitates their docking [40–42]. Alternatively, PKA could directly phosphorylate Cx40, since all three endothelial connexins, Cx43, Cx40 and Cx37, possess putative phosphorylation sites for PKA [12,43–45]. It has been shown that Cx43 phosphorylation was increased by forskolin in rat granulosa cells [46]. However, treatment with 8-Br-cAMP did not alter Cx43 phosphorylation in MDCK cells [47]. Correspondingly, no effect on permeability and conductance of Cx43 was found after 8-Br-cAMP incubation [48]. Concerning Cx37 or Cx40, no concisive data with respect to phosphorylation by PKA exist. Phosphorylation might be involved in the constitutive transport of Cx40 to the plasma membrane and its functional incorporation. PKA might also have effects on the microtubuli, which are needed to translocate connexins from intracellular stores as recently demonstrated [49,50]. Finally, it can also be speculated that PKA facilitates lateral diffusion of Cx in the plasma membrane, thus enhancing the assembly of gap junction plaques [51]. Since this last mechanism would, in contrast to our findings (Fig. 7), not have led to redistribution of Cx from intracellular stores to the plasma membrane, it seems rather unlikely.

In conclusion, NO enhances de novo formation of endothelial gap junctions, which is specific for Cx40 and involves PKA activation. This effect is accompanied by cGMP-dependent enhancement of cAMP levels. The resulting activation of PKA leads to an increased incorporation of Cx40 into the plasma membrane. This novel action of NO on endothelial gap-junctional coupling may be of particular interest in the context of angiogenesis [3,4], as NO plays an important role as an angiogenic factor modulating migration and sprouting of endothelial cells.

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