Critical Role of Cx40 in Reduced Endothelial Electrical Coupling by Lipopolysaccharide and Hypoxia-Reoxygenation

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Key Words

Endothelial cells · Electrical coupling · Cx40 · Acute inflammation

Abstract

Background: We discovered that lipopolysaccharide (LPS, an initiating factor in sepsis) and hypoxia-reoxygenation (H/R, a confounding factor) reduce electrical coupling between microvascular endothelial cells from wild-type (WT) but not Cx40^{−/−} mice. Because Cx40 knockout could result in nonspecific effects, this discovery may not establish the causal relationship between Cx40 and reduced coupling. Using the same cell culture model, we aimed to address this uncertainty by using the rescue-of-function approach. **Methods/Results:** Electrical coupling between endothelial cells (hind-limb muscle origin) was determined by electrophysiology. LPS, H/R and concurrent LPS + H/R reduced coupling between WT but not Cx40^{−/−} cells. The defect in Cx40^{−/−} cells was rescued by ectopic expression of Cx40, after infecting the cells with adenovirus encoding Cx40. Cx40^{−/−} cells were also engineered to express mutant Cx40 that lacked the carboxyl terminal domain beginning at residue 236 (Cx40_{Δ237–358}) or 344 (Cx40_{Δ345–358}). No response to inflammatory stimuli was observed in cells expressing either of these 2 mutants. **Conclusion:** Our data establish the causal relationship between

Cx40 and reduced coupling and suggest that the 345–358 amino acid motif of the Cx40 carboxyl terminal is required for reduced coupling. Cx40 may participate in compromised conducted response in the microvasculature during sepsis.

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Introduction

Sepsis is a systemic inflammatory response to local infection. We have shown that sepsis attenuates the arteriolar conducted response, thus contributing to hemodynamic failure [1, 2]. The response involves the passage of electrical currents along the endothelial layer of the arteriole via interendothelial gap junctions [3]. To explain the attenuated conduction, we used electrophysiology and monolayers of cultured microvascular endothelial cells (MMEC, of mouse skeletal muscle origin) to discover that lipopolysaccharide (LPS, an initiating factor in sepsis) and hypoxia-reoxygenation (H/R, a confounding factor) reduce electrical coupling in cells from wild-type (WT), Cx37^{−/−} and Cx43^{G60S} ‘non-functional’ mutants, but not from Cx40^{−/−} mice (review [4]). These results suggested a causal relationship between Cx40 and reduced coupling after exposure to the inflammatory stimuli. However, because MMEC express a low level of Cx40 protein [5], it

was not clear how Cx40 dictates the response to the stimuli in the presence of other more abundant connexins.

It has been reported that Cx43 knockout in mice affects the expression of numerous proteins [6]. Cx40 knockout can result in nonspecific effects [7]. To address this issue, the main objective of this study was to use the 'rescue-of-function' approach in Cx40^{-/-} cells, by reintroducing Cx40 into the cells by infecting them with an adenovirus encoding Cx40. If the loss of response to the stimuli were due to a nonspecific effect, then introduction of Cx40 would not rescue the response. A further objective was to probe for a critical motif of Cx40 responsible for the response. To this end, we engineered Cx40^{-/-} cells to express 2 mutants of Cx40 that lacked the specific carboxyl terminal (CT) domains of Cx40. We hypothesized that either deletion mutant may eliminate the anticipated rescue of response in Cx40^{-/-} cells.

Methods

Reagents, mouse strains, isolation and culturing of MMEC, electrophysiological approach (i.e. injection of 2–4 hyperpolarizing constant-current pulses of 25 nA and 100 ms duration at 1-second intervals into cell monolayers), treatment with LPS, H/R and concurrent LPS + H/R as well as quantitative PCR, immunofluorescence and Western blot analyses were all based on our published reports [5, 8, 9], and are therefore included in the electronic supplement (Methods; for all online suppl. material, see www.karger.com/doi/10.1159/000445772).

Recombinant Adenovirus

Adenoviruses (AdV) carrying cDNA of β -galactosidase (AdV- β -gal), WT Cx40 (AdV-Cx40) and Cx40 truncated at CT residues 236 (AdV-Cx40 $_{\Delta 237-358}$) and 344 (AdV-Cx40 $_{\Delta 345-358}$) were separately cloned and supplied by Applied Biological Materials Inc. (Vancouver, B.C., Canada). The viruses were amplified using a standard procedure using human embryonic kidney 293 cells. They were purified and titrated with the adenovirus purification mega kit and adenovirus titer immunoassay kit, respectively, following the manufacturer's instructions. We applied Adv-Cx40, Adv-Cx40 $_{\Delta 237-358}$ or Adv-Cx40 $_{\Delta 345-358}$ to MMEC harvested from Cx40^{-/-} mice (70–75% confluent monolayers) at a multiplicity of infection 50 IFU/cell, and further incubated for 24 h. AdV- β -gal was used in parallel as a negative control. Successful infection was determined by detection of Cx40 mRNA and Cx40 protein expression in infected Cx40^{-/-} cells.

Statistical Analysis

The data are presented as mean $\pm$ standard error. MMEC were isolated from at least 3 different mice (n indicates the number of monolayers used per treatment group, unless otherwise stated). Statistical significance was assessed using ANOVA and two-tailed Student's t test with Bonferroni's correction, unless otherwise stated. Significance was set to $p < 0.05$.

Results

Cell Morphology and Baseline Resting Membrane Potential

LPS (10 μ g/ml, 1 h), H/R (0.1% O₂ for 1 h, followed by 5–20 min reoxygenation), or LPS + H/R stimuli did not result in any visual morphological changes in the appearance of the cell monolayers. Resting membrane potential (E_m) values for Cx40^{-/-}, Cx40^{-/-} + AdV-Cx40, Cx40^{-/-} + AdV-Cx40 $_{\Delta 237-358}$, Cx40^{-/-} + AdV-Cx40 $_{\Delta 345-358}$ and WT cells were -12.7 ± 4.1 (n = 22), -13.2 ± 6.0 (n = 16), -12.7 ± 3.3 (n = 8), -14.1 ± 4.2 (n = 11) and -13.5 ± 6.5 mV (n = 6), respectively. The values did not differ between groups. The values were less than those reported in vivo or ex vivo preparations [10], but they fell within the range reported for cultured endothelial cells [11, 12]. Resting E_m values remained unchanged after cell exposure to the inflammatory stimuli.

Effect of LPS, H/R and LPS + H/R on Coupling in Cx40^{-/-} and WT Cells

To assess coupling, we used a method of constant-current pulse injection into the cell monolayer (electronic supplement, Methods; a simple model of the method is shown in online suppl. fig. S1). In practice, we measured current-induced E_m deflections mostly at 50-, 150- and 250- μ m distances from the injection electrode (fig. 1). The largest deflections occurred at 50 μ m (summarized in online suppl. fig. S2). From the spatial decay of these deflections, we computed the intercellular resistance (r_i). The legend to figure 1 provides examples of computed r_i for both Cx40^{-/-} and Cx40^{-/-} + AdV-Cx40 cells under control and LPS conditions. Figure 2a summarizes percent r_i changes from control for Cx40^{-/-}, Cx40^{-/-} + AdV-Cx40 and WT cells exposed to LPS, H/R and LPS + H/R. The stimuli did not affect r_i in Cx40^{-/-} cells, but they increased r_i (i.e. reduced electrical coupling) in the Cx40^{-/-} + AdV-Cx40 and WT cells. Thus, ectopically expressed Cx40 in Cx40^{-/-} cells rescued the response to these stimuli. The transmembrane resistivity in Cx40^{-/-} cells (9.5 ± 5.9 ; n = 22), Cx40^{-/-} + AdV-Cx40 cells (11.5 ± 7.6 ; n = 16) and WT cells (7.6 ± 1.7 k Ω cm²; n = 6) was not altered by the inflammatory stimuli. The data for Cx40^{-/-} and WT cells (fig. 2a) showing a larger effect of LPS + H/R when compared to LPS or H/R alone agree with our previous work with these cells [9].

Figure 2b summarizes r_i data from Cx40^{-/-} cells expressing either of the 2 Cx40 mutants. LPS did not affect r_i in Cx40^{-/-} + AdV-Cx40 $_{\Delta 237-358}$ or Cx40^{-/-} + AdV-Cx40 $_{\Delta 345-358}$ cells. LPS did not affect r_i in Cx40^{-/-} +

(For legend see next page.)

AdV- β -gal cells (legend to fig. 2b). Similarly, H/R did not affect r_i in Cx40^{-/-} + AdV-Cx40 $_{\Delta 237-358}$ or Cx40^{-/-} + AdV-Cx40 $_{\Delta 345-358}$ cells. Thus, the mutants failed to rescue the response to these stimuli, suggesting a critical role of Cx40 residues 345–358 in the response. The transmembrane resistivity in Cx40^{-/-} + AdV-Cx40 $_{\Delta 237-358}$ cells (9.6 ± 5.2 , $n = 8$) and Cx40^{-/-} + AdV-Cx40 $_{\Delta 345-358}$ cells (10.1 ± 6.5 k Ω cm², $n = 11$) was not altered by the stimuli.

Detection of Cx40 mRNA

Based on the real-time PCR in Cx40^{-/-} cells, infected Cx40^{-/-} cells and WT cells, and on measured Ct values for β -actin and Cx40 mRNA expressions in these cells, the average fold increases of Cx40 mRNA expression in Cx40^{-/-} + AdV-Cx40, Cx40^{-/-} + AdV-Cx40 $_{\Delta 237-358}$ and Cx40^{-/-} + AdV-Cx40 $_{\Delta 345-358}$ cells were 22.0, 17.5 and 12.2, respectively, relative to the Cx40 mRNA expression in WT cells (online suppl. fig. S3). No Cx40 mRNA was detected in the Cx40^{-/-} cells.

Detection of Cx40 Protein

Using the anti-Cx40 antibody targeting Cx40CT (Alpha Diagnostics), no Cx40 protein was detected by immunofluorescence in the Cx40^{-/-} cells, as expected (fig. 3a). In contrast, Cx40 protein plaques were detected with the same antibody in Cx40^{-/-} + AdV-Cx40 cells (fig. 3b), indicating successful infection with AdV-Cx40 (presumably, some of the Cx40 plaques would be gap junction plaques). Cx40 protein was also detected with

this antibody in the WT cells, but to a lesser extent than in the infected cells (fig. 3c). The low number of Cx40 protein plaques in the WT cells was consistent with our previous report of a low level of Cx40 protein expression in WT MMEC probed by immunofluorescence [5]. No Cx40 protein was detected in a negative control experiment in the WT cells where the anti-Cx40 antibody was omitted (data not shown).

Next, we used the Invitrogen anti-Cx40 antibody (i.e. the only available antibody not requiring Cx40CT for Cx40 detection) to probe for Cx40 in Cx40^{-/-} + AdV-Cx40 $_{\Delta 345-358}$ cells. The performance of this antibody was disappointing. Unlike the Alpha Diagnostics antibody showing no Cx40 protein in the Cx40^{-/-} cells, it showed nonspecific staining in the Cx40^{-/-} cells (fig. 3d). Despite this drawback, it did detect Cx40 protein plaques in Cx40^{-/-} + AdV-Cx40 $_{\Delta 345-358}$ cells (fig. 3e), indicating successful infection with AdV-Cx40 $_{\Delta 345-358}$. Cx40 plaques were also detected with this antibody in the WT cells (fig. 3f), but to a lesser extent than the infected cells.

To extend our immunofluorescence results, we used Western blotting to show that WT cells infected with AdV-Cx40 express Cx40 protein. We used the Zymed anti-Cx40 antibody (the Alpha Diagnostics and Invitrogen antibodies did not work). Online supplementary figure S4 shows negligible Cx40 protein expression in WT cells, but noticeable expression in WT + AdV-Cx40 cells. The low/negligible Cx40 presence in putative gap junction plaques in WT cells was confirmed with immunofluores-

Fig. 1. Examples of electrophysiological recordings in microvascular endothelial cells obtained from hind-limb skeletal muscle of Cx40^{-/-} mice. **a** A situation for the control Cx40^{-/-} cell monolayer where a cell was impaled by a current-injecting electrode E_1 (not shown). Subsequently, a recording electrode E_2 impaled another cell in the monolayer at a 50- μ m interelectrode distance at the time marked by the step change in membrane potential E_{m2} from 0 to -12 mV. Next, electrode E_1 injected 3 brief constant-current pulses into the first cell, causing brief superimposed E_{m2} deflections of -10 mV. The step change in E_{m2} to zero marks the time of electrode E_2 withdrawal from the cell. The procedure was repeated for interelectrode distances of 150 and 250 μ m, and E_{m2} deflections for all distances were used to compute r_i (an inverse measure of electrical coupling) as detailed in Electronic Supplement, Methods. **b** Examples of E_{m2} recordings from Cx40^{-/-} monolayer exposed to LPS (10 μ g/ml for 1 h). **c** A plot of E_{m2} deflections of **a** and **b** against the log of interelectrode distance. Lines represent the best fit through the points. Based on these plots, r_i was computed as 1.6 and 1.4 M Ω for the control LPS conditions, respectively. **d** Lines of best fit of E_{m2} deflections from all experiments in **c**. There was no difference

in slopes between the control and LPS conditions. $n = 35$ deflections (1 deflection/each impaled cell) at 50- μ m interelectrode distance, 1 at 75 μ m, 30 at 150 μ m, 2 at 175 μ m, 31 at 250 μ m, 1 at 275 μ m and 2 at 300 μ m for the control, and 14 at 50 μ m, 1 at 75 μ m, 19 at 150 μ m, 2 at 175 μ m, 14 at 250 μ m, 1 at 275 μ m and 1 at 300 μ m for LPS in Cx40^{-/-} cells. **e, f** Examples of E_{m2} recordings from Cx40^{-/-} + AdV-Cx40 monolayer subjected to control and LPS conditions. **g** A plot of E_{m2} deflections of **e** and **f** against the log of interelectrode distance. We computed $r_i = 1.5$ and 4.3 M Ω for the control and LPS conditions, respectively. **h** Lines of best fit of E_{m2} deflections from all experiments exemplified in **g**. The slope for the LPS condition was significantly larger when compared to the control, demonstrating that LPS increased the slope only in the Cx40^{-/-} + AdV-Cx40 monolayer, i.e. when Cx40 was ectopically expressed in Cx40^{-/-} cells. * $p < 0.05$ compared to control, using the GraphPad program for comparisons between slopes, $n = 29$ deflections at 50 μ m, 1 at 75 μ m, 4 at 100 μ m, 1 at 125 μ m, 30 at 150 μ m, 4 at 200 μ m, 28 at 250 μ m and 2 at 300 μ m for the control, and 35 at 50 μ m, 2 at 75 μ m, 4 at 100 μ m, 1 at 125 μ m, 25 at 150 μ m, 4 at 200 μ m, 21 at 250 μ m and 2 at 300 μ m for LPS in Cx40^{-/-} + AdV-Cx40 cells.

Fig. 2. Effect of inflammatory stimuli on r_i in mouse microvascular endothelial cells. We used LPS (10 $\mu\text{g/ml}$, for 1 h), hypoxia (0.1% O_2 for 1 h) and reoxygenation (5–20 min), i.e. H/R, and concurrent LPS + H/R. **a** Percentage changes in r_i with respect to control r_i are shown for Cx40^{-/-}, Cx40^{-/-} + AdV-Cx40, and WT cells. Control r_i values: $1.4 \pm 0.3 \text{ M}\Omega$, $1.5 \pm 0.3 \text{ M}\Omega$ and $1.5 \pm 0.6 \text{ M}\Omega$ in Cx40^{-/-} cells, $1.3 \pm 0.5 \text{ M}\Omega$, $0.9 \pm 0.1 \text{ M}\Omega$, and $1.2 \pm 0.5 \text{ M}\Omega$ in Cx40^{-/-} + AdV-Cx40 cells and $1.3 \pm 0.3 \text{ M}\Omega$, $1.3 \pm 0.2 \text{ M}\Omega$ and $1.3 \pm 0.2 \text{ M}\Omega$ in WT cells before stimulation with LPS, H/R and LPS + H/R, respectively. * $p < 0.05$ compared to the respective control group, $n = 4\text{--}22$ cell monolayers per group. **b** Percentage changes in r_i are shown for Cx40^{-/-} + AdV-Cx40_{Δ237-358} and Cx40^{-/-} + AdV-Cx40_{Δ345-358} cells. Control r_i values: $1.6 \pm 0.2 \text{ M}\Omega$ and $1.5 \pm 0.2 \text{ M}\Omega$ in Cx40^{-/-} + AdV-Cx40_{Δ237-358} cells and $1.4 \pm 0.3 \text{ M}\Omega$ and $1.6 \pm 0.3 \text{ M}\Omega$ in Cx40^{-/-} + AdV-Cx40_{Δ345-358} cells before stimulation with LPS and H/R, respectively ($n = 4\text{--}6$ per group). For Cx40^{-/-} + AdV- β -gal cells, the percent change after LPS was $94 \pm 6\%$ of the control value. The control r_i was $1.5 \pm 0.3 \text{ M}\Omega$ ($n = 6$ per group). Inflammatory stimuli increased r_i (i.e. reduced electrical coupling) only in Cx40^{-/-} cells infected with AdV-Cx40 and in WT cells.

cence using Zymed and Chemicon antibodies, in both untreated and LPS-treated cells (online suppl. fig. S5). Taken together, infection with AdV-Cx40 increased Cx40 protein expression in Cx40^{-/-} cells when examined by immunofluorescence (compare fig. 3a, b) as well as in WT cells when examined by Western blot (online suppl. fig. S4).

Discussion

This study shows, for the first time, that the introduction of WT Cx40 into Cx40^{-/-} microvascular endothelial cells rescues the reduction in electrical coupling initiated by LPS, H/R and LPS + H/R, thus establishing a causative link between Cx40 and the reduction. Moreover, our data show that the introduction of Cx40 without the residues 345–358 does not rescue the reduction, suggesting that this Cx40CT domain is required for the reduced coupling.

Methodological Considerations

Two issues are considered. First, there was a methodological difficulty in accurately determining the baseline r_i (i.e. control r_i). This determination depends on a number of parameters (e.g. monolayer confluency and thickness, number of gap junctions between adjacent cells and connexin composition of gap junctions). Because the accurate determination of all these parameters was technically very difficult on an experimental day-to-day basis, the study was designed in the following way. For each cell line prepared for a particular experimental day, we passaged cells to an appropriate number of monolayer-covered coverslips. Then, on the experimental day, we randomly assigned coverslips to the control or treatment group (electronic supplement, Methods). This procedure permitted a comparison between r_i in the control and treatment groups within a cell line, but no comparison between baseline r_i among different cell lines studied on different days. Thus, because of the technical difficulty in controlling all parameters required for computing the baseline r_i , our results did not lend themselves to establish an effect of Cx40 knockout and Cx40 infection on baseline r_i .

The second issue is the low Cx40 protein expression seen in WT cells and in cells infected with AdV-Cx40 (fig. 3; online suppl. fig. S4). Cx40 protein is abundantly expressed in the endothelium of mammalian microvasculature in vivo [13]. However, exposure of endothelial cells to culturing conditions may profoundly diminish their protein expression. For example, cell passaging caused a marked loss of CD14 protein in HUVEC [14]. In cultured microvascular endothelial cells obtained from rat skeletal muscle, we detected a high level of Cx40 mRNA, but no Cx40 protein [15]. Posttranslational modifications associated with cell culturing and/or compensation by other connexins as well as the poor performance of commercial Cx40 antibodies, could have contributed to this lack of detection. The loss of protein in

Fig. 3. Cx40 protein distribution in Cx40^{-/-}, Cx40^{-/-} + AdV-Cx40, Cx40^{-/-} + AdV-Cx40_{Δ345-358} and WT endothelial cells at semiconfluence. **a–c** Representative examples of cells probed with an anti-Cx40 antibody (Alpha Diagnostics) targeting the CT of Cx40. Punctate staining (arrows) denotes Cx40 protein plaques. The superimposed oval uniform gray shapes represent cell nuclei identified with Hoechst 33342 stain; n = 2 (Cx40^{-/-}), 3 (Cx40^{-/-} + AdV-Cx40) and 7 (WT). **d–f** Representative examples of cells probed

with an anti-Cx40 antibody targeting the cytoplasmic loop of Cx40 (Invitrogen). Punctate staining (arrows) denotes Cx40 protein plaques, while oval uniform gray shapes represent cell nuclei; n = 13 (Cx40^{-/-}), 15 (Cx40^{-/-} + AdV-Cx40_{Δ345-358}), and 7 (WT). **b, e** Dashed lines represent approximate cell borders. **d–f** The nonpunctate staining was judged as nonspecific antibody background staining. Bar = 20 μm.

the cultured cells and the poor antibody performance also hampered the findings of this study. This included the low level of Cx40 protein in WT cells, and, in view of the high level of Cx40 mRNA expression, the less-than-expected level of Cx40 protein in cells infected with AdV-Cx40.

Importance of Cx40 in Intercellular Coupling

Cx40 is required for the microvascular conducted response in vivo [16, 17]. This in vitro study indicates that Cx40 also plays a modulatory role in electrical coupling in response to acute inflammatory stimuli. We reported that, in cultured WT MMEC, expression of large-size gap junction plaques of Cx43 protein dominates over Cx37 and Cx40 protein expression [5]. Cx43 may therefore be

responsible for the baseline electrical coupling in these cells. Based on our evidence of loss of function by Cx40 knockout and restoration of function by the reintroduction of Cx40, it is intriguing that Cx40 may play a modulatory role in coupling during exposure to inflammatory stimuli. To this end, we suggest that Cx40 and Cx43 may be intermixing. Indeed, Cx40/Cx43 heteromeric channels have been shown to be more sensitive to stimulation than Cx40 or Cx43 homomeric channels [18, 19]. Since Cx40/Cx43 heteromeric channels are formed in vascular cells [20], the stimulus-sensitive Cx40 constituent of these channels present even as a minor constituent could be responsible for the modulatory role [21]. Alternatively, Cx40 could participate in this modulation, independently of its role in gap junctional intercellular communication, possibly by affecting the function of other gap junctional connexins indirectly [22]. Thus, the location of Cx40 protein within the cell (i.e. the cell membrane vs. the cytosol; fig. 3b) may not be critical to the response to LPS and H/R observed in this study. Examination of the potential role of Cx40/Cx43 heteromeric channels and/or the indirect role of Cx40 during exposure to inflammatory stimuli was beyond the scope of the study.

Our results suggest that Cx40CT is required for the modulation of coupling. Cx40CT has been shown to play a critical role in the responses of homomeric Cx40 and heteromeric Cx40/Cx43 channels to various stimuli [18, 23]. We reported that the response to inflammatory stimuli in WT cells is PKA-dependent [9, 11]. Cx40 contains a consensus sequence for PKA [24] where the C-terminal serine residues 345, 348, 349 and 353 are 4 possible PKA phosphorylation sites [25]. The lack of response here in Cx40^{-/-} + AdV-Cx40_{Δ345-358} cells is consistent with the possibility that these residues are important for the modulation.

Several mechanisms could explain the stimuli-induced reduction in coupling. If this reduction involved heteromeric Cx40/Cx43 channels, then changes in internalization of these channels, the number of gap junctions remaining at the cell-cell surface or ZO-1 binding to Cx40 [26] could all affect intercellular coupling. However, because the stimuli-induced responses in r_i occur rapidly (within 4–30 min) and because r_i is also restored rapidly after the removal of the stimuli [15, 27], the gap junction electrical behavior (i.e. altered gap junction conductance) may best explain the observed changes in r_i .

In conclusion, in mouse microvascular endothelial cells, we show that LPS, H/R and LPS + H/R reduced electrical coupling in WT but not Cx40^{-/-} cells, and that this reduction was rescued by introducing Cx40 into the Cx40^{-/-} cells. If the loss of reduction were caused by a nonspecific effect of Cx40 knockout, then the introduction of Cx40 could not rescue the reduction. Introduction of Cx40 without the residues 345–358 did not rescue the reduction, suggesting that these residues play an important role in the reduction of coupling.

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Disclosure Statement

There are no disclosures.

References

- Lidington D, Ouellette Y, Li F, Tyml K: Conducted vasoconstriction is reduced in a mouse model of sepsis. *J Vasc Res* 2003;40:149–158.
- Tyml K, Wang X, Lidington D, Ouellette Y: Lipopolysaccharide reduces intercellular coupling in vitro and arteriolar conducted response in vivo. *Am J Physiol Heart Circ Physiol* 2001;281:H1397–H1406.
- Emerson GG, Segal SS: Electrical activation of endothelium evokes vasodilation and hyperpolarization along hamster feed arteries. *Am J Physiol Heart Circ Physiol* 2001;280:H160–H167.
- Tyml K: Role of connexins in microvascular dysfunction during inflammation. *Can J Physiol Pharmacol* 2011;89:1–12.
- McKinnon RL, Bolon ML, Wang HX, Swarbreck S, Kidder GM, Simon AM, Tyml K: Reduction of electrical coupling between microvascular endothelial cells by NO depends on connexin37. *Am J Physiol Heart Circ Physiol* 2009;297:H93–H101.
- Iacobas DA, Scemes E, Spray DC: Gene expression alterations in connexin null mice extend beyond the gap junction. *Neurochem Int* 2004;45:243–250.
- Briset AC, Isakson BE, Kwak BR: Connexins in vascular physiology and pathology. *Antioxid Redox Signal* 2009;11:267–282.
- Swarbreck S, Secor D, Li F, Gross PL, Ellis CG, Sharpe MD, Wilson JX, Tyml K: Effect of ascorbate on fibrinolytic factors in septic mouse skeletal muscle. *Blood Coagul Fibrinolysis* 2014;25:745–753.
- Bolon ML, Peng T, Kidder GM, Tyml K: Lipopolysaccharide plus hypoxia and reoxygenation synergistically reduce electrical coupling between microvascular endothelial cells by dephosphorylating connexin40. *J Cell Physiol* 2008;217:350–359.
- He P, Curry FE: Measurement of membrane potential of endothelial cells in single perfused microvessels. *Microvasc Res* 1995;50:183–198.

- 11 Bolon ML, Ouellette Y, Li F, Tyml K: Abrupt reoxygenation following hypoxia reduces electrical coupling between endothelial cells of wild-type but not connexin40 null mice in an oxidant- and PKA-dependent manner. *FASEB J* 2005;19:1725–1727.
- 12 Vargas FF, Caviedes PF, Grant DS: Electrophysiological characteristics of cultured human umbilical vein endothelial cells. *Microvasc Res* 1994;47:153–165.
- 13 Gustafsson F, Mikkelsen HB, Arensbak B, Thuneberg L, Neve S, Jensen LJ, Holstein-Rathlou NH: Expression of connexin 37, 40 and 43 in rat mesenteric arterioles and resistance arteries. *Histochem Cell Biol* 2003;119:139–148.
- 14 Jersmann HP, Hii CS, Hodge GL, Ferrante A: Synthesis and surface expression of CD14 by human endothelial cells. *Infect Immun* 2001;69:479–485.
- 15 Lidington D, Ouellette Y, Tyml K: Endotoxin increases intercellular resistance in microvascular endothelial cells by a tyrosine kinase pathway. *J Cell Physiol* 2000;185:117–125.
- 16 de Wit C, Roos F, Bolz SS, Kirchhoff S, Kruger O, Willecke K, Pohl U: Impaired conduction of vasodilation along arterioles in connexin40-deficient mice. *Circ Res* 2000;86:649–655.
- 17 Wang L, Yin J, Nickles HT, Ranke H, Tabuchi A, Hoffmann J, Tabeling C, Barbosa-Sicard E, Chanson M, Kwak BR, Shin HS, Wu S, Isakson BE, Witzentrath M, de Wit C, Fleming I, Kuppe H, Kuebler WM: Hypoxic pulmonary vasoconstriction requires connexin 40-mediated endothelial signal conduction. *J Clin Invest* 2012;122:4218–4230.
- 18 Stergiopoulos K, Alvarado JL, Mastroianni M, Ek-Vitorin JF, Taffet SM, Delmar M: Hetero-domain interactions as a mechanism for the regulation of connexin channels. *Circ Res* 1999;84:1144–1155.
- 19 Gu H, Ek-Vitorin JF, Taffet SM, Delmar M: Ultrarapid communication: coexpression of connexins 40 and 43 enhances the pH sensitivity of gap junctions: a model for synergistic interactions among connexins. *Circ Res* 2000;86:1100.
- 20 He DS, Jiang JX, Taffet SM, Burt JM: Formation of heteromeric gap junction channels by connexins 40 and 43 in vascular smooth muscle cells. *Proc Natl Acad Sci USA* 1999;96:6495–6500.
- 21 Burt JM, Steele TD: Selective effect of PDGF on connexin43 versus connexin40 comprised gap junction channels. *Cell Commun Adhes* 2003;10:287–291.
- 22 Yu G, Bolon M, Laird DW, Tyml K: Hypoxia and reoxygenation-induced oxidant production increase in microvascular endothelial cells depends on connexin40. *Free Rad Biol Med* 2010;49:1008–1013.
- 23 Anumonwo JM, Taffet SM, Gu H, Chanson M, Moreno AP, Delmar M: The carboxyl terminal domain regulates the unitary conductance and voltage dependence of connexin40 gap junction channels. *Circ Res* 2001;88:666–673.
- 24 Blom N, Gammeltoft S, Brunak S: Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* 1999;294:1351–1362.
- 25 Hjerrild M, Stensballe A, Rasmussen TE, Kofoed CB, Blom N, Sicheritz-Ponten T, Larsen MR, Brunak S, Jensen ON, Gammeltoft S: Identification of phosphorylation sites in protein kinase A substrates using artificial neural networks and mass spectrometry. *J Proteome Res* 2004;3:426–433.
- 26 Bouvier D, Kieken F, Kellezi A, Sorgen PL: Structural changes in the carboxyl terminus of the gap junction protein connexin 40 caused by the interaction with c-Src and zonula occludens-1. *Cell Commun Adhes* 2008;15:107–118.
- 27 Rose K, Ouellette Y, Bolon M, Tyml K: Hypoxia/reoxygenation reduces microvascular endothelial cell coupling by a tyrosine and MAP kinase dependent pathway. *J Cell Physiol* 2005;204:131–138.