Gap junction inhibitors reduce endothelium-dependent contractions in the aorta of spontaneously hypertensive rats

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List of abbreviations: COX: cyclooxygenase; DETCA: diethyldithiocarbamic acid; EDRF: endothelium-derived contracting factor; ROS: reactive oxygen species; SHR: spontaneously hypertensive rat; SOD: superoxide dismutase; TP receptor: thromboxane prostanoid receptor; WKY: Wistar Kyoto rat.

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Abstract:

Experiments were designed to determine the effect of gap junction inhibitors on endothelium-dependent contractions. Isolated aortic rings of spontaneously hypertensive rats (SHR) were suspended in vitro for isometric force recording. The non-selective gap junction inhibitor, carbenoxolone reduced endothelium-dependent contractions to acetylcholine and the calcium ionophore A23187. There was no or modest effect of the gap peptides ⁴⁰Gap27, ³⁷,⁴³Gap27 or ⁴³Gap26, when applied alone on endothelium-dependent contractions. However the combined treatment with the three gap peptides significantly decreased endothelium-dependent contractions. The combined inhibition of the three connexins was not as effective as carbenoxolone, suggesting the involvement of other connexins in the process of endothelium-dependent contraction. The present study shows the involvement of gap junctions in endothelium-dependent contractions of the SHR aorta, presumably that of the combination of connexins 37, 40, 43 rather than a single subtype of these proteins. Contractions of the vascular smooth muscle caused by U46619 and prostacyclin, but not to those of endoperoxides and phenylephrine were reduced only minimally by carbenoxolone. Thus, if gap junction signaling is involved in the contraction of the vascular smooth muscle to thromboxane-prostanoid (TP) receptor agonists, their contribution is small. This suggests that the reduction of endothelium-dependent contractions by carbenoxolone and the gap peptides cannot be attributed to the homocellular gap junctions between vascular smooth muscle, but more likely involves the homocellular gap junctions between endothelial cells and/or myoendothelial gap junctions.
Introduction

The generation of reactive oxygen species (ROS) by xanthine plus xanthine oxidase causes endothelium-independent contractions of the rat aorta that are augmented in preparations of spontaneously hypertensive (SHR) compared to normotensive Wistar Kyoto (WKY) rats (Auch-Schwelk et al., 1989; Yang et al., 2002). The contraction is blocked by TP receptor antagonists, indomethacin, valeryl salicylate [preferential COX-1 inhibitor], but not by NS-398 [preferential COX-2 inhibitor] (Auch-Schwelk et al., 1989; Yang et al., 2002). Thus, the SHR smooth muscle is more sensitive to ROS and activation of its COX-1 and TP receptors are vital for the response (Yang et al., 1998; Hibino et al., 1999; Yang et al., 2002; Rodriguez-Martinez et al., 1998; Gil-Longo & Gonzalez-Vazquez, 2005).

Endothelium-dependent contractions of the rat aorta are prevented by inhibitors of cyclooxygenase (Lüscher & Vanhoutte 1986; Ge et al., 1995; Gluais et al., 2005; 2006). Superoxide anions are an endothelium-derived contracting factor (EDCF) in canine basilar (Katusic & Vanhoutte, 1989; Katusic et al., 1993) and the rat renal (Gao & Lee, 2005) arteries. In the SHR aorta, superoxide dimutase (SOD, which dismutates superoxide anions into oxygen and hydrogen peroxide), catalase (which decomposes hydrogen peroxide to water and oxygen), or deferoxamine (which prevents the formation of hydroxyl radicals) do not affect endothelium-dependent contractions (Auch-Schwelk et al., 1989). Because of their large molecular size, the poor intracellular penetration of these scavengers may explain their ineffectiveness (Auch-Schwelk et al., 1989; Yang et al., 2002). This interpretation is hard to reconcile with the finding that SOD prevents endothelium-dependent contractions in the canine basilar artery (Katusic &
Vanhoutte, 1989) and in aortas of rats treated chronically with dimethylthiourea (in vivo hydroxyl radical scavenger) (Yang et al., 2002). In the SHR aorta, tiron (an intracellular scavenger of superoxide anions) and diethyldithiocarbamic acid (DETCA; an inhibitor of SOD) reduced endothelium-dependent contractions in an additive manner (Yang et al., 2002). Measurement of ROS with ROS-sensitizing fluorescence dyes revealed that EDCF-mediated responses are accompanied by a burst of endothelial ROS (Tang et al., 2007). The production of endothelial ROS is augmented in the aorta of SHR compared to that of the WKY (Tang et al., 2007). The ROS presumably were derived directly from the endothelial cyclooxygenase, since this endothelial production of ROS in response to acetylcholine was prevented by indomethacin (Tang et al., 2007). Thus, ROS contribute to endothelium-dependent contractions, but whether or not they are the EDCF itself or act as facilitators is uncertain.

The amplitude of endothelium-dependent contractions observed in “sandwich” layered preparations is smaller than that observed in intact aortic rings (Yang et al., 2003). In such layered “sandwich” preparations, endothelium-dependent contractions are likely to be mediated largely by diffusion of relatively stable molecules such as prostanoids with minimal contribution of ROS, because of the expanded intercellular gap between endothelial and smooth muscle cells and the short half life of the free radicals. In such “sandwich” preparations, unlike in intact rings, the combination of SOD plus catalase reduced the endothelium-dependent response to acetylcholine (Yang et al., 2003), suggesting, that in intact preparations the transfer of ROS from the endothelial cells to the underlying vascular smooth muscle is through shielded channels. The transfer of endothelium-dependent hyperpolarizing signals (EDHF) through myoendothelial gap junctions is well established (Griffith et al., 2004; Feletou and Vanhoutte 2007). Myoendothelial
gap junctions may provide a continuity transfer for ROS in the intact aorta. Obviously, in layered “sandwich” preparations myoendothelial gap junctions are ripped apart and the ROS produced by the endothelial cells are spilled out into the widened intercellular gap where they become accessible to the scavenging by SOD and catalase, despite the poor cell permeability of these two compounds.

The present study was designed to investigate whether or not endothelium-dependent contractions in the SHR aorta involve the transfer of ROS through myoendothelial gap junctions, which would explain why the response is uninterrupted by antioxidants with poor cellular permeability in intact preparations.
Methods

Tissue preparation

SHR were maintained under a 12h light/12 dark cycle at 21±1°C and fed with standard laboratory chow and water ad libitum. They were anesthetized with pentobarbital sodium (70 mg/ml/kg; Ganes Chemicals Inc. Pennsville, NJ, USA). Their thoracic aorta was quickly excised and placed in Krebs-Ringer bicarbonate buffer of the following composition (mM): NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25 and glucose 11.1 (control solution). The aortae were cleaned of adherent connective tissue and were cut into rings (approximately 3mm in length) for the measurement of isometric tension. The present investigations were approved by the committee on the use of live animals in teaching and research of University of Hong Kong.

Isometric tension:

Aortic rings from 36-45 weeks old SHR were equilibrated in organ chambers (Tang et al., 2005, 2008). To study endothelium-dependent contractions, quiescent rings were incubated with Nω-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor; 10^-4M) for 40 minutes, to optimize endothelium-dependent contractions (Auch-Schwelk et al., 1992; Yang et al., 2004; Tang et al., 2005). They then were exposed to progressively increasing concentrations of acetylcholine (10^-9 to 10^-4M) or the calcium ionophore A23187. To study responses of the smooth muscle only, the endothelium was removed mechanically by inserting the tip of a syringe needle into the ring and rolling them back and forth in a sylgard-based container filled with control solution. After equilibration, the rings without endothelium were exposed to either a
single dose or to progressively increasing concentrations of the contractile agent studied. Some preparations were treated with carbenoxolone (a non-selective gap junction inhibitor, $10^{-4}$M), diethyldithiocarbamate acid (DETCA, inhibitor of SOD; $10^{-5}$M), a gap peptide [either with $37,43$Gap27 (a selective connexin [Cx] 37 and 43 inhibitor), $40$Gap27 (a selective Cx40 inhibitor) or $43$Gap26 (a selective Cx43 inhibitor), at $3x10^{-4}$M each] or exposed to the combined treatment of three gap peptides ($37,43$Gap27 + $40$Gap27 + $43$Gap26; $3x10^{-4}$M each; with a final concentration totaling to $9x10^{-4}$M of gap peptides) for 50 minutes before evoking contractions. All changes in tension were expressed as percentage of the reference contraction to 60 mM KCl obtained at the start of the experiment.

**Drugs**

Acetylcholine, the calcium ionophore A23187, carbenoxolone, diethyldithiocarbamate acid (DETCA), Nω-nitro-L-arginine methyl ester (L-NAME), phenylephrine (PE) and tiron were purchased from Sigma Chemical Company (St Louis, MO, USA). Iloprost, prostaglandin I$_2$ sodium salt (prostacyclin) and prostaglandin H$_2$ were purchased from Cayman Chemicals (Ann Arbor, MI, USA). AnalaR® grade potassium chloride (KCl) was purchased from VWR International (Poole, England). The gap peptides $37,43$Gap27 (H-Ser-Arg-Pro-Thr-Glu-Lys-Thr-Ile-Phe-Ile-Ile-OH), $40$Gap27 (H-Ser-Arg-Pro-Thr-Glu-Lys-Asn-Val-Phe-Ile-Val-OH), $43$Gap26 (H-Val-Cys-Tyr-Asp-Lys-Ser-Phe-Pro-Ile-Ser-His-Val-Arg-OH)] were purchased from Biomatik Corporation (Wilmington, DE, USA). U46619 was purchased from Biomol (Plymouth Meeting, PA., U.S.A). A stock solution of calcium ionophore A23187 was prepared in absolute DMSO. A stock solution of indomethacin was prepared in a sodium bicarbonate ($5x10^{-3}$ M) solution. A stock solution of U46619 was prepared in absolute ethanol. All other compounds
were prepared in deionized water. Concentrations are expressed as final molar concentrations in the bath solution.

**Data Analysis**

The results are presented as means ± standard error of the mean (SEM) with n being the number of individual observations in aortas from different rats. Data were analyzed using the statistical program, Prism version 3a (GraphPad Software; San Diego California; USA). Paired Student’s t-test for comparison of two groups or one-way ANOVA with repeated measures followed by the Bonferroni post-test were carried out for multiple comparisons. A difference was accepted as statistically significant when probability (P) values were less than 0.05.
Results

Acetylcholine and the calcium ionophore A23187 evoked contraction in a concentration-dependent manner in aortic rings of the SHR. These contractions were reduced significantly when the endothelial layer of the aortic rings was removed (Figure 1a,b). Carbenoxolone at $10^{-4}$M, a dose which is effective and commonly used to inhibit gap junction communication (Edwards et al., 1999; Tracey et al., 2002; Dora et al., 2003), significantly reduced the endothelium-dependent contractions to acetylcholine and A23187 (Figure 1c,d).

The endothelium-dependent contractions to A23187 were not affected significantly by the gap peptides $37,43\text{Gap}27$, $40\text{Gap}27$ or $43\text{Gap}26$ (at $3 \times 10^{-4}$M) given alone. The latter two gap peptides did not affect endothelium-dependent contraction to acetylcholine either (Figure 2). However, $37,43\text{Gap}27$ alone significantly reduced the contraction evoked by acetylcholine. The endothelium-dependent contractions to both acetylcholine and A23187 were reduced significantly by the combined treatment with three gap peptides (at $3 \times 10^{-4}$M each) (Figure 2).

The endothelium-dependent contractions evoked by acetylcholine and the calcium ionophore A23187 were concentration-dependently reduced by the antioxidant, DETCA (a cell permeable inhibitor of SOD) (Figure 3a,b). However, tiron (a cell permeable superoxide anion scavenger) at $10^{-2}$M or $10^{-3}$M did not significantly alter the level of endothelium-dependent contraction (Figure 3c,d). The combined treatment with DETCA ($10^{-5}$M) with carbenoxolone further inhibited endothelium-dependent contractions to acetylcholine and A23187 (Figure 4).
U46619 (a synthetic TP receptor agonist), prostacyclin, phenylephrine, KCl, prostaglandin H₂, but not iloprost, evoked endothelium-independent contractions in aortic rings of SHR without endothelium (Figure 5). Carbenoxolone (10⁻⁴M), but not DETCA (10⁻⁵M) significantly shifted the concentration-response curve to U46619 and prostacyclin to the right (Figure 5a, d). Both carbenoxolone and DETCA significantly reduced the contraction to concentrations of KCl greater than 50mM (Figure 5e). Carbenoxolone and DETCA did not significantly affect the contractions to either phenylephrine or prostaglandin H₂ (Figure 5b, c).
Discussion

The non-selective gap junctions inhibitor, carbenoxolone reduced, but did not abolish endothelium-dependent contractions. This novel finding suggests that communication via gap junctions is involved in endothelium-dependent contractions. Gap junctions are composed of two connexons, which are themselves constructed out of six connexin (Cx) molecules. Cx37, Cx40 and Cx43 are the main connexins expressed in vascular tissues (Larson et al., 1990; Beyer et al., 1992; Reed et al., 1993; Rummery & Hill, 2004). This prompted the use of specific gap peptides targeted against each of these connexins in the present study. The gap peptides $^{40}\text{Gap}27$ or $^{43}\text{Gap}26$ prevent the docking of Cx40 and Cx43, respectively and $^{37,43}\text{Gap}27$ inhibits Cx37 and Cx43 simultaneously (Chaytor et al., 2001; Ujiie et al., 2003). Incubation with the individual gap peptides failed to reduce endothelium-dependent contractions, except for $^{37,43}\text{Gap}27$, which caused a small but significant reduction of the endothelium-dependent contraction to acetylcholine, presumably owning to its dual inhibitory effect on two separate connexins. The most effective inhibition by gap peptides was achieved by the combined treatment with the three peptides, suggesting that Cx37, Cx40, Cx43 all are involved in endothelium-dependent contractions to acetylcholine and A23187. Taken as a whole, the results suggest that multiple connexins are involved in EDCF-mediated responses. The inhibition achieved by the combination of the three individual gap peptides is not as marked as those produced by the non-selective gap junction inhibitor, carbenoxolone. This suggests that other connexins, such as connexin 45 (Li & Simard, 2001) or other unidentified connexins (Rummery & Hill, 2004) may be expressed and contribute to endothelium-dependent contraction in the rat aorta. Carbenoxolone also inhibits steroid receptors (Sewell et al., 1998) and since these are present on
vascular smooth muscle cells (Mhailidou & Funder, 2005), this effect may contribute to the depression of endothelium-dependent contractions observed in the present study with the compound.

Recent reports have suggested that unpaired connexons (also termed hemichannels) exist and function similarly to gap junction channels, displaying low substrate selectivity and permitting the transfer of molecules with molecular masses smaller than 1 kDa (Cherian et al., 2005; Spray et al., 2006). Most inhibitors of gap junction, such as the one used in the present study also inhibit hemichannels. However, based on the observations that cell-permeable antioxidants, such as DETCA but not antioxidants of poor cellular permeability (Auch-Schwelk et al., 1989, Yang et al., 2002) reduce endothelium-dependent contractions, it is unlikely that ROS are released through hemichannels during endothelium-dependent contractions since opened hemichannels would allow the release of ROS from the cell interior into the extracellular space, which should be prone to the attack by poor permeability antioxidants. Hemichannels have been proposed to be involved in the release of prostaglandins in cultured osteocytes (Cherian et al., 2005). However, the present study on intact tissues did not investigate whether prostaglandins traverses the plasma membrane of endothelial cells through hemichannels during endothelium-dependent contraction.

The cell permeable SOD inhibitor, DETCA effectively reduced the endothelium-dependent contractions to acetylcholine and the calcium ionophore A23187, confirming the role of ROS in these responses of the SHR aorta. The inhibitory effect of DETCA on endothelium-dependent contraction was concentration-dependent. At the highest contraction tested, $10^{-4}$M, DETCA
virtually prevented endothelium-dependent contractions, suggesting that ROS are not just partial contributors to the response but that their presence is critical for the process. The ineffectiveness of SOD and catalase shown in previous studies (Auch-Schwelk et al., 1989) in affecting endothelium-dependent contraction is probably due to their low cellular permeability (Auch-Schwelk et al., 1989; Yang et al., 2002). Even for drugs that are able to cross the membrane effectively, it is possible that it may not reach the right compartment within the cell to cause their actions, which could explain the ineffectiveness of tiron to reduce endothelium-dependent contractions. The present findings differ from those reported earlier (Yang et al., 2002), demonstrating that both tiron (albeit minimally) and DETCA reduced endothelium-dependent contractions independently and that the effects of the two antioxidants were additive. The reason for this discrepancy between the two studies is unknown, but it may reflect differences in experimental setting or in the origin of tiron. DETCA did not alter responsiveness of rings without endothelium to U46619, phenylephrine, prostaglandin H₂, prostacyclin. Thus the ability of DETCA to reduce endothelium-dependent contractions suggests an endothelial site of action, rather than an effect on vascular smooth muscle.

The addition of DETCA to carbenoxolone-treated preparations, in which communication through gap junctions is prevented, further reduced the level of endothelium-dependent contractions. This observation suggests that ROS not only are transferred through gap junctions, but presumably also affect contractions in a way independent of gap junction communication. One possibility could be that ROS are transferred from cell to cell through passive diffusion or alternatively that ROS may facilitate the vasoconstrictor action of diffusible vasoconstrictor prostaglandins.
The association of connexins between adjacent endothelial cells or neighboring vascular smooth muscle forms homocellular gap junctions. Connexins can also form heterocellular gap junctions, such as those connecting endothelial and smooth muscle cells (myoendothelial gap junctions). The present study illustrates a role of gap junction in endothelium-dependent contractions, but the location of the gap junctions involved in EDCF-mediated responses is uncertain. To address this, whether or not the direct contraction of smooth muscle requires communication through gap junctions was tested. Both selective TP receptor agonists (responsible for EDCF-mediated contractions) and non-TP receptor agonists were used. The result show that TP receptor-mediated contractions to U46619 and prostacyclin of the vascular smooth muscle of the SHR aorta partially requires communication through gap junctions, as indicated by the ability of carbenoxolone to reduce the increase in tension evoked by these agonists. The effect of carbenoxolone is not universal for all TP receptor activated contractions, as it did not affect the contraction to prostaglandin H2. This difference in sensitivity to gap junction inhibition with different TP receptor agonists may reflect the presence of different isoforms of TP receptors in the rat aorta, as has been suggested in the human (Miggin & Kinsella, 1998) or different structural organization of TP receptors such as homo- or hetero-dimerization of TP receptors (Wilson et al., 2004). In support of this hypothesis, the sensitivity to prostaglandin H2 vascular smooth muscle is higher in the SHR compared to WKY, but there is no difference in the sensitivity to U46619 between the aortae of the two strains (Ge et al., 1995). Since carbenoxolone inhibited minimally the contraction of KCl and did not affect the response to phenylephrine, its ability to reduce contraction appears to be specific to only certain TP receptor agonists. In the present study, iloprost, the synthetic prostacyclin analogue did not cause contraction, presumably because this drug was designed as a selective activator of relaxant IP
receptor (Abramovitz et al., 2000) and thus may not cross react with contractile TP receptors as is the case of authentic prostacyclin (Gluais et al., 2005).

The effectiveness of carbenoxolone in inhibiting endothelium-dependent contractions to both acetylcholine and A23187, with no or only modest effects on contractions to prostaglandin H\textsubscript{2} and prostacyclin, respectively [which are the two main candidates of EDCF in the SHR aorta] (Ge et al., 1995; Gluais et al. 2005; Tang et al., 2008), suggests that gap junctions other than the homocellular gap junctions between smooth muscle cells contribute to endothelium-dependent contractions. The present findings cannot differentiate whether the communication through gap junctions involved in endothelium-dependent contractions is specifically due to myoendothelial gap junction, homocellular gap junction between endothelial cells or both. Likewise, the present findings do not provide direct evidence that ROS can be transferred through gap junctions. However the current demonstration that gap junctions are involved in the process opens the possibilities that myoendothelial gap junctions may act as a shielded channel for the transfer of EDCF from the endothelial cells to the underlying smooth muscle. The present study thus strengthens the hypothesis that endothelium-dependent contractions of the SHR aorta are due to two components: a) formation of endothelium-derived, cyclooxygenase-dependent prostanoids that directly activate TP receptors on the vascular smooth muscle; and b) formation of endothelial ROS that presumably are channeled through the myoendothelial gap junctions and stimulate the cyclooxygenase in the vascular smooth muscle to further produce prostanoids activating their TP receptors.
References


Footnotes

This work is supported by the RGC of HKU
Legends for figures:

Figure 1: Endothelium-dependent contraction to a) acetylcholine and b) the calcium ionophore A23187 in isolated SHR aortic rings. The effect of carbenoxolone (a non-selective gap junction inhibitor; 10^{-4}M) on endothelium-dependent contraction to c) acetylcholine and d) calcium ionophore A23187. N = 5; +EC = with endothelium; -EC; without endothelium; * P < 0.05 vs +EC.

Figure 2: Effect of gap peptides (300mM) on endothelium-dependent contractions to acetylcholine (left) and the calcium ionophore A23187 (right). Three gap peptides = 37,43Gap27 + 40Gap27 + 43Gap26 (at 300mM each; a total concentration of 900mM); +EC = with endothelium; N = 5; *P < 0.05 vs. +EC.

Figure 3 Effect of antioxidants on endothelium-dependent contractions to acetylcholine (a,c) and the calcium ionophore A23187 (b,d). +EC = with endothelium; diethyldithiocarbamate acid (DETCA; 10^{-5} or 10^{-4} M; a cell permeable SOD inhibitor); Tiron (10^{-3} or 10^{-2} M; a cell permeable superoxide anion scavenger). N =5; *P < 0.05 vs +EC.

Figure 4 Combined effect of diethyldithiocarbamate acid (DETCA; 10^{-5}M) plus carbenoxolone (10^{-4}M) on endothelium-dependent contractions to acetylcholine (left) and the calcium ionophore A23187 (right). N =5; +EC = with endothelium; *P < 0.05 vs. +EC; #P < 0.05 vs. carbenoxolone.
Figure 5 Effects of carbenoxolone (10^{-4}M) and diethylthiocarbamate acid (DETCA; 10^{-5}M) on contractions of rat aortic rings without endothelium to U46619, prostaglandin H2 (PGH2), phenylephrine (PE), prostacyclin (PGI2), potassium chloride (KCl) and iloprost. To study the contraction mediated by prostaglandin H2, it was applied to aortic rings at a single concentration of 10^{-6}M, owing to its instability in control solution. All other agonists were applied in a cumulative manner. N=5, +EC; with endothelium; *P < 0.05 vs. +EC.
Figure 4

Changes in Tension (% of 60mM KCl)

Acetylcholine (Log M)

A23187 (Log M)

- +EC
- Carbenoxolone
- DETCA+Carbenoxolone

Changes in Tension (% of 60mM KCl)

0 - 20 - 40 - 60 - 80 - 100

-7 -6 -5 -4

-7.5 -7.0 -6.5 -6.0 -5.5

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