Development of the myogenic response in postnatal intestine: role of PKC

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Su, Baogen Y., Kristina M. Reber, Craig A. Nankervis, and Philip T. Nowicki. Development of the myogenic response in postnatal intestine: role of PKC. Am J Physiol Gastrointest Liver Physiol 284: G445–G452, 2003; 10.1152/ajpgi.00259.2002.—Previous attempts to determine developmental changes in the vascular myogenic response have been confounded by the presence of competing vasoactive stimuli or the use of isolated vessels with markedly different baseline diameters. To circumvent these issues, small mesenteric arteries (diameter ~150 μm) from 1- and 10-day-old piglets were studied in vitro under no-flow conditions. In situ studies demonstrated that the intravascular pressure and diameter of these vessels were similar in both age groups, allowing an effective comparison of the myogenic response not obscured by differences in basal diameter. The pressure-diameter relationship was age specific. Thus, although small mesenteric arteries from both age groups demonstrated myogenic constriction in response to stepwise increases in pressure (0 to 100 mmHg, in 20-mmHg increments), the intensity of contraction was significantly greater in vessels from 1-day-old piglets particularly within the pressure range normally experienced by these vessels in situ. Attenuation or activation of PKC with calphostin C or indolactam, respectively, substantially altered the pressure-diameter relationship in 1-, but not 10-day-old arteries; thus calphostin C essentially eliminated the contractile response to pressure elevation in younger subjects, whereas indolactam significantly increased the intensity of the myogenic response and shifted its activation point to a lower pressure range. Immunoblots carried out on protein recovered from these arteries revealed the presence of α, β, ε, γ, and λ; notably, expression of the α- and ε-isozymes substantially decreased between postnatal days 1 and 10. newborn intestine; intestinal circulation

The mechanistic basis of the myogenic response is calcium-dependent activation of the actin-myosin motor unit, as evidenced by a brisk rise in VSM intra-cellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) in response to the mechanostimulus of pressure and by subsequent phosphorylation of myosin light chain kinase (3, 22, 34). However, other factors also play a role in the initiation and maintenance of myogenic vasoconstriction, reviewed most recently by Davis and Hill (5); of these, that which has direct bearing on the present work is PKC (6, 13, 15, 16, 19, 27). For example, pressure stimulation applied to coronary arterioles caused a transient increase in [Ca$^{2+}$], but a sustained translocation of PKCα from cytosol-to-membrane in VSM, indicating its activation (6). It was suggested that PKCα enhanced the Ca$^{2+}$-sensitivity of VSM by phosphorylation and thus disinhibition of the myosin binding proteins calponin and caldesmon, facilitating maintenance of the pressure-induced arteriolar contraction despite reduction of [Ca$^{2+}$]; (10, 24, 32).

The myogenic response is present within the postnatal intestinal circulation; however, it is not clear whether significant changes in the intensity of this response occur during early postnatal development. Studies carried out within in situ gut loops prepared in 1- to 35-day-old swine suggested that the magnitude of vasoconstriction in response to an acute elevation of mesenteric venous pressure, the perturbation used to elicit the myogenic response within the entire gut circulation, was greater in older subjects (2, 26). Interpretation of these data was confounded by the presence of myriad vasoactive stimuli present in the whole organ preparation. For example, the absence of vasoconstriction in response to venous pressure elevation in 1-day-old subjects was attributed to a vasodilatory metabolic feedback signal (derived from the parenchyma) overriding myogenic vasoconstriction (2). Studies (23, 29) carried out in small mesenteric arteries mounted in vitro suggested the opposite developmental pattern, i.e., that the magnitude of the myogenic response was greater in younger subjects. However, the baseline diameters of the vessels that comprised the two age groups were significantly different. This discrepancy...
could be important, because the intensity of pressure-induced myogenic vasoconstriction is proportional to the vessel diameter (4). Thus the difference in the magnitude of pressure-induced vasoconstriction could have reflected the different starting diameters, rather than a difference on the basis of developmental changes in the myogenic response.

The goal of these experiments was to reevaluate the developmental features of the myogenic response within the postnatal intestinal circulation by using an experimental format that circumvented the confounding variables present in earlier studies. To this end, the myogenic response was studied in vitro in small mesenteric arteries (diameter ~150 μm) harvested from 1- and 10-day-old swine. These age groups were chosen because the intravascular pressures and diameters of these arteries from these age groups are similar under in situ conditions. The putative role of PKC in generation and maintenance of myogenic vasoconstriction to increased transmural pressure was evaluated by activation of PKC with indolactam or attenuation of its function with calphostin C. As well, immunoblots were carried out to determine the presence of specific PKC isoforms in homogenates prepared from mesenteric arteries.

METHODS

Animal Acquisition and Handling

Two age groups of postnatal swine were studied: 1 and 10 days old. Age ranges were not used in this study. Hence, subjects in the 1-day-old group were consistently ≥ 18 ≤ 24 h old at the time of actual study, whereas those in the older group were studied on postnatal day 10. All subjects were farm-reared at a single commercial swine farm where they were kept with the sow and allowed to feed ad libitum until the time of transport to the lab. Subjects were not fasted before surgical preparation; therefore, the stomachs of all subjects were distended with milk curd at the time of surgery before surgical preparation; therefore, the stomachs of all subjects were kept with the sow and allowed to feed ad libitum until old at the time of actual study, whereas those in the older age group. Intra-arterial pressures within these arteries were measured in anesthetized, ventilated, 1- and 10-day-old piglets. Saline-filled glass micropipettes, tip diameter 40 μm, were inserted into the vessels with the aid of a micromanipulator and dissecting microscope, and connected to a low-compliance pressure transducer. Separate arteries were impaled proximal to their origin at the arterial plexus, at midvessel, or at a distal site, just before their penetration of the intestinal wall (i.e., each artery was impaled at only 1 site). In all instances the micropipette was <40% of the luminal diameter of the artery. These measurements were carried out in five subjects in each age group; in all instances, these subjects were also used to harvest small mesenteric arteries for the immunoblot studies described below (harvested arteries were different from those used for the pressure measurements).

In Situ Studies of Small Mesenteric Arteries

Preparation. Arteries were removed from the mesentery of the distal jejunum and proximal ileum and mounted in the proper proximal-distal orientation between two glass micropipettes seated within a plastic chamber (Living Systems, Burlington, VT). The inflow pipette was fixed, whereas the outflow pipette was mounted on a micrometer to allow adjustment along the long axis of the vessel that was secured in place by 11-0 ophthalmic suture. Perfusion was achieved by using oxygenated Krebs buffer of the following composition (in mM): 118.1 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, 11.1 glucose, and 0.026 EDTA, pH 7.4 when gassed with 16% O2-5% CO2-balance N2 at 37°C. The perfusion circuit was designed as a "blind sac," i.e., the outflow circuit was closed to the artery once buffer had filled the arterial lumen. Pressure within the artery was regulated by a pressure transducer driven by a servo-controlled pump. This system allowed rapid and precise changes in pressure within the artery in the absence of intraluminal flow. This approach was chosen to study the myogenic response to changes in pressure inside the mesenteric artery in the absence of flow-induced mechanostimuli (29). The vessel chamber, and thus the exterior surface of the artery was continuously suffused with warmed, oxygenated Krebs buffer at a rate of 50 ml/min. The suffusate was continuously recirculated (total volume 200 ml). The vessel chamber was mounted on the stage of an inverted microscope set in line with a video camera. Vascular dimensions were measured with a precalibrated video dimension analyzer (V94; Living Systems) that displayed wall thickness and intraluminal diameter.

Experimental protocols. In all instances the mounted artery was initially pressurized to 40 mmHg and allowed 30–45 min to reach a steady-state diameter. During this time, all functional arteries demonstrated a significant reduction in diameter (i.e., >20% reduction from the initial diameter when pressurized to 40 mmHg); arteries that did not demonstrate this spontaneous constriction were discarded. A similar percentage of arteries were discarded in both age groups.
Pressure was then increased to 45 mmHg, and the average midvessel pressure noted under in situ conditions. The arteries either contracted or maintained their diameter in response to this pressure challenge, indicating a myogenic response to the increase in pressure. The suffusion buffer was switched to 40 mM KCl-Krebs buffer, which elicited a brisk contraction in all arteries. Substance P (10^{-8} M) was added to the suffusion buffer to verify the presence of an intact endothelial layer, inasmuch as this peptide activates the endothelial isoform of NOS in swine intestinal endothelial cells (25). Vessels that did not contract >50% to KCl or dilate >25% to substance P were discarded. A similar percentage of arteries were discarded from both age groups. The buffers were replaced with fresh Krebs and the pressure was left at 40 mmHg. Thereafter, pressure was reduced to 0 mmHg and then increased to 100 mmHg in progressive increments of 20 mmHg (pressure ramp). Each pressure was maintained until the vessel diameter attained a new steady-state value. A second pressure ramp was then carried out after the addition of either vehicle (dimethylsulfoxide), indolactam (0.1 μM), or calphostin C (1.0 μM) to the suffusion buffer and then after exchanging the suffusion buffer with a Ca^{2+}-free buffer containing 1 mM EGTA to determine the passive response of the vessels to stepwise pressure increases. The doses of indolactam and calphostin C were selected based on pilot studies in which the effects of increasing drug concentrations on vessel diameter were noted at a pressure of 50 mmHg.

Three additional protocols were also carried out. First, studies were carried out in arteries from 1-day-old animals to confirm that prior administration of calphostin C attenuated the effect of indolactam, i.e., that the selected PKC blocking agent could decrease the effect of the selected PKC stimulating agent. Second, because calphostin C has the potential to affect L-type Ca^{2+} channels, studies were carried out in which the potassium concentration in the superfusion buffer was increased in the presence of calphostin C to ensure that the contractile response to membrane depolarization was not affected by calphostin C. Finally, because initial experiments demonstrated that the effects of indolactam on vessel diameter were greater in 1- than 10-day-old animals and that expression of PKCα and -ε was also greater in younger arteries, we carried out studies in arteries from 10-day-old animals to determine whether a dose-response relationship was present.

Western Blotting

Terminal mesenteric arteries (TMAs) were removed from study subjects used for the in situ pressure experiments and were frozen by immersion into liquid N_2. Frozen tissue was vortexed for 10 min, centrifuged, and then the supernatant was recovered. Protein separation was carried out by electrophoresis in 7% SDS-polyacrylamide gels (10 μg protein/lane) and then transferred overnight to polyvinylidene difluoride membranes. After blocking for 1 h in 5% dried milk in PBS/Tween buffer, the membranes were incubated with primary antibody for the classic PKC isoforms (α, β1, β2, γ), the novel PKC isoforms (δ, ε, θ, η), or the atypical PKC isoforms (ζ, λ, ξ). All antibodies were obtained from Transduction Laboratories except for ζ, which was obtained from GIBCO. Primary antibody was diluted in PBS/Tween buffer with 1% milk (all 1:500) and was applied for 1 h at room temperature. Thereafter, membranes were rinsed, incubated with horse-radish peroxidase conjugated secondary antibody (1:2,500; Calbiochem) for 1 h, and then developed by using enhanced chemiluminescence from Pierce. Membranes were then stripped and reprobed with primary antibody for β-actin to confirm equal protein loading in all lanes.

Data Analysis

Myogenic responsiveness of the vessels was determined by expressing vessel diameter in the presence of Ca^{2+} to the diameter under passive conditions (Ca^{2+} absent, EGTA added) at the same pressure. Comparisons of the pressure-diameter relationships were similar for the control portion of the studies for all four treatment groups within each age group. The control data for each age group were therefore pooled for the initial statistical comparison of control data between age groups (Fig. 1). This comparison was carried out by using a two-way ANOVA for repeated measures that utilized age group (i.e., not the pooled control data) (Figs. 4 and 5). These comparisons were carried out by using a three-way ANOVA for repeated measures that utilized age group, treatment (drug vs. control), and pressure as main effects. The F-statistic for both ANOVAs was significant (P < 0.01); thereafter, post hoc Tukey’s B tests were carried out to determine the sites of significance, again accepting significance at the P < 0.01 level. Western blots for the PKCα and -ε isoforms were scanned, and the densitometry data was evaluated by unpaired t-tests to determine developmental differences in PKC expression in small mesenteric arteries from 1- and 10-day-old animals.
RESULTS

The intravascular pressures within small mesenteric arteries from 1- and 10-day-old subjects were similar under in situ conditions. In 1-day-old animals, pressures within the proximal, midpoint, and distal portions of the arteries were 50 ± 2, 43 ± 2, and 36 ± 2 mmHg. Pressures within similar portions in arteries from 10-day-old animals were 51 ± 3, 42 ± 3, and 36 ± 2, respectively. A substantial pressure drop from the proximal-to-distal portion of the artery was present under in situ conditions in both age groups (14 ± 2 mmHg in 1-day-old, 15 ± 2 mmHg in 10-day-old subjects), confirming that these vessels function as part of the resistance vasculature in postnatal swine intestine. The diameters of the small mesenteric arteries were similar in both age groups: 152 ± 13 microns in 1-day-old vs. 164 ± 14 µm in 10-day-old, respectively.

Despite their similar in situ diameters and pressure characteristics, the behavior of these vessels when pressurized under in vitro conditions was age specific (Fig. 1). Arteries from 1-day-old subjects demonstrated a significant decrease in vessel diameter when pressure was increased above the average in situ pressure of 42 mmHg, whereas vessels from 10-day-old subjects did not. Arteries from the 10-day-old subjects maintained their diameter when pressure was increased to 60 and then to 80 mmHg, i.e., they did not behave in a passive manner; yet, their response was clearly not as abrupt as that noted in the 1-day-old arteries.

The effects of calphostin C (1 µM), a selective PKC antagonist, were age specific. The contractile response to pressure was significantly attenuated by calphostin C in arteries from 1-day-old animals (Fig. 2). Treatment with calphostin C caused small mesenteric arteries from 1-day-old animals to dilate in response to pressure increments in a manner nearly identical to that caused by eliminating Ca^{2+} from the perfusion and suffusion buffers. In contrast to the dramatic change noted in younger subjects, arteries from 10-day-old subjects failed to demonstrate a significant change in the pressure-diameter relationship after pretreatment with calphostin C, but did demonstrate completely passive behavior in response to incremental increases in intravascular pressure when Ca^{2+} was removed from the buffers (Fig. 3). The effects of calphostin C were specific to its action on PKC; thus application of calphostin C did not effect the contractile response to membrane depolarization induced by increasing the KCl concentration within the suffusate buffer.

Response of small mesenteric arteries to activation of PKC by indolactam was also age-specific. Indolactam (0.1 µM) significantly increased the contractile response to pressure at all pressures >0 mmHg in TMAs from 1-day-old subjects and also shifted the initiation of the response to a lower pressure level (Fig. 4). In contrast, indolactam did not exert a significant effect on the contractile response to pressure in arteries from 10-day-old animals (Fig. 5). However, the addition of greater concentrations of indolactam to the superfusate buffer in arteries from 10-day-old animals increased their contractile response to pressure. Thus in the presence of 1.0 µM indolactam, pressurization of 10-day arteries to 40 and 60 mmHg led to an increased contraction, such that the ratio of active to passive...
diameter decreased to $0.43 \pm 0.03$ and $0.42 \pm 0.05$, respectively, a level similar to that observed in 1-day-old arteries. Pretreatment of arteries with calphostin C significantly attenuated the effect of indolactam on the response of 1-day-old arteries to step increases in pressure.

Immunoblots carried out on homogenates of small mesenteric arteries revealed the presence of five PKC isoforms in both age groups: $\alpha$, $\beta$, $\epsilon$, $\iota$, and $\lambda$. (Fig. 6). Of these, a clear reduction in expression of the $\alpha$ and $\epsilon$ isoforms was noted between postnatal days 1 and 10, whereas no change in the $\beta$-, $\iota$-, or $\lambda$-isoform was noted (Fig. 7). Densitometry was carried out on the blots for the PKC$\beta$ and $\epsilon$ isoforms. The intensity of PKC$\beta$, expressed as a function of $\beta$-actin, was $87 \pm 5$ and $37 \pm 4\%$ for mesenteric arteries from 1- and 10-day-old subjects, respectively ($P < 0.01$ by unpaired $t$-test). The intensity of PKC$\epsilon$ was $32 \pm 3$ and $17 \pm 3\%$ for mesenteric arteries from 1- and 10-day-old subjects, respectively ($P < 0.01$ by unpaired $t$-test).

**DISCUSSION**

The Myogenic Response Is Age Dependent in Small Mesenteric Arteries

The first novel observation made in these experiments is that the relationship between intravascular pressure and vessel diameter in small mesenteric arteries is age specific in 1- and 10-day-old piglets. Ves-
Vessels from 1-day-old subjects demonstrated a significant reduction in diameter when pressure was increased above the average pressure normally experienced by these vessels in situ, i.e., they displayed a sharp myogenic response. In contrast, arteries from 10-day-old subjects failed to demonstrate a significant reduction in diameter in response to pressure elevation, but instead maintained diameter over the pressure range normally experienced by this vessel in situ. It is important to note that the lack of a reduction in diameter in these vessels does not imply absence of myogenic response to pressure elevation; indeed, comparison of Figs. 1 and 5 clearly demonstrates that these vessels demonstrated contraction in response to stepwise pressure elevation, inasmuch as the pressure-diameter curves generated under control vs. Ca²⁺-free conditions were quite different. Rather, it can be concluded that the intensity of the myogenic response is age dependent, being more substantial in younger subjects.

The design of this study eliminated many of the confounding variables that plagued previous studies of the myogenic response to pressure elevation in postnatal intestine (2, 26). Observations were made in isolated arteries (i.e., vessels that were devoid of surrounding parenchyma) thus eliminating potential interference by the putative metabolic feedback signal. This signal, generated by parenchymal elements in response to their level of oxygen sufficiency, functions as a vasodilating stimulus designed to augment oxygen transport and thus ensure that the oxygen supply/demand ratio remains favorable (12). This vasodilation could clearly interfere with the observation of myogenic vasoconstriction. As well, the studies were conducted under “no-flow” conditions, i.e., flow within the artery lumen was absent, achieved by keeping the pressures at the inlet and outlet micropipettes precisely equal. This arrangement prevented the generation of flow-induced dilation, a process wherein the mechanostimulus of flow stimulates the endothelial isofrom of NOS to augment nitric oxide production (28). We (23, 29) have previously demonstrated that flow-induced dilation is present in small mesenteric arteries from 1-day-old animals and that it dramatically attenuates the myogenic response to intravascular pressure elevation. Finally, the diameters of 1- and 10-day-old arteries are similar, both under in situ conditions and when set at a pressure of 0 mmHg in vitro. Davis (4) demonstrated that the intensity of the myogenic response is inversely proportional to the diameter of the vessel. Thus our prior observations regarding developmental differences in the myogenic response of these vessels from 1- and 40-day-old subjects could have reflected the significant difference in the basal diameters of these vessels at these postnatal ages (23, 29).

What might be the physiological relevance of postnatal transition in the intensity of the myogenic response? The fetal intestine is functionally dormant compared with the newborn intestine and has a high vascular resistance, most likely because its oxidative demands are significantly less (7). In this context, it might be anticipated that mechanisms would be in place designed to enhance vascular tone and thus enhance resistance within the fetal intestinal circulation, such as an intensified myogenic response. The requirement for an increased basal vascular resistance across the intestine changes dramatically at birth, however, as increased intestinal function demands an enhanced oxygen delivery. Hence, the need for an intensified myogenic response is eliminated after parturition. Although this notion is speculative in nature, it is logical to expect some type of transitional physiology within the intestinal circulation during the emergence from fetal life, as clearly occurs in the other dormant fetal organ, the lung (33).

PKC Plays a Role in the Myogenic Response in Small Mesenteric Arteries from 1-Day-Old Subjects

The second novel observation made in these experiments was the age-specific participation of PKC in modulating the intensity of the myogenic response. Attenuation of PKC activity with the selective antagonist calphostin C (20) virtually eliminated the myogenic response in small mesenteric arteries from 1-day-old subjects, whereas stimulation of PKC activity with indolactam (14) significantly increased the degree of contraction in response to stepwise increases in intravascular pressure. In contrast to these effects, these agents failed to have a significant impact on the pressure-diameter relationship in small mesenteric arteries from 10-day-old subjects. A possible explanation for this age-specific response was found in the immunoblots; thus expression of the α- and ε-isoforms of PKC were substantially greater in small mesenteric arteries from 1- than from 10-day-old subjects.

The initial reports of the involvement of PKC in modulating the myogenic response were on the basis of the effects of relatively nonspecific PKC antagonists (H7, staurosporine) on hemodynamic regulation within the microvasculature (15, 21). These observations followed studies that demonstrated significant changes in the myogenic response by α-agonists, such as norepinephrine, inasmuch as it was established that α1 receptor binding enhanced the production of diacylglycerol, one of several agents necessary to activate the classic isoforms of PKCα, -β, and -γ (8). Later work by Osol et al. (27) and Karibe et al. (19) confirmed a role for PKC in the myogenic response by using a more specific PKC antagonist, calphostin C (20). These observations were pivotal in establishing a key role for PKC in modulating the myogenic response because H7 and staurosporine function by binding to the catalytic domain of PKC, a domain shared by many kinases. Thus earlier observations could have reflected attenuation of myosin light chain kinase function, rather than just PKC (15, 21, 30). In contrast, calphostin C functions by binding to the regulatory domain of PKC, an area not shared by other kinases, making the agent highly specific for PKC function (20). Subsequent work
has suggested that the effect of PKC on vascular tone is independent of changes in VSM [Ca2+], (10, 32). Instead, PKC appears to function by phosphorylation of myosin binding proteins, such as caldesmon and calponin; this action attenuates the inhibitory effect of these proteins on the actin-myosin motor unit and thus increases the Ca2+ sensitivity of the contractile apparatus, i.e., enhancing the degree of contraction achieved at a given VSM [Ca2+], (10, 24, 32).

The fact that expression of the α- and ε-isozymes of PKC decrease between postnatal days 1 and 10 is certainly not sufficient to conclude that these isozymes were responsible for the changes in the pressure-diameter relationships noted in small mesenteric arteries from 1-day-old animals after calphostin C or indolactam. Indeed, although these agents are specific for PKC, they are not selective in their function vis-a-vis a specific isoform (14, 20). However, it is important to note that published reports have linked these isoforms with vascular regulation, particularly in modification of VSM contraction in response to a pressure or stretch stimulus (1, 6, 13, 16). Thus Horowitz et al. (16) applied the PKCζ and -ζ isoforms to saponin-permeabilized VSM and noted the induction of contraction by the ε isoform only, a contraction that was ablated by the PKC pseudosubstrate inhibitor peptide PKC19–31. Later work from the same laboratory demonstrated translocation of the α-isozyme of PKC in ferret coronary arterioles in response to a pressure stimulus (6). In this context, translocation of PKCα occurs as part of its activation, so that demonstration of PKCα translocation implies activation of the enzyme in response to the stretch stimulus provided by pressure increase.

In summary, three novel observations were made in these experiments. The pressure-diameter relationships of small mesenteric arteries from 1- and 10-day-old piglets are different when studied in vitre, under no-flow conditions; thus the magnitude of the myogenic contraction to stepwise pressure elevation is present in both age groups, but is more substantial in the 1-day-old group. Blockade or activation of PKC with calphostin C or indolactam, respectively, significantly changes the pressure-diameter relationship of small mesenteric arteries from 1-, but not 10-day-old subjects in a manner suggesting that PKC modulates the myogenic response in younger subjects. Finally, protein expression of the PKCα and ε isoforms substantially decreases between postnatal days 1 and 10. We conclude that the intensity of the myogenic response in small mesenteric arteries from postnatal intestine is developmentally regulated and speculate that developmental expression of PKC, particularly PKCε and PKCs, may partake in this phenomenon.

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