Ceramide in the antiapoptotic effect of ischemic preconditioning

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Submitted 7 July 2003; accepted in final form 26 August 2003

Argaud, Laurent, Annie-France Prigent, Lara Chalabreysse, Joseph Loufouat, Michel Lagarde, and Michel Ovize. Ceramide in the antiapoptotic effect of ischemic preconditioning. Am J Physiol Heart Circ Physiol 286: H246–H251, 2004.—Although the mechanism by which ischemic preconditioning (PC) inhibits myocardial apoptosis during ischemia-reperfusion is unclear, evidence indicates a role for the secondary messenger ceramide. We investigated in vivo whether PC may affect ceramide and sn-1,2-diacylglycerol (DAG) production, and attenuate apoptosis during ischemia. Rabbids underwent 30 min of ischemia, followed by 4 h of reperfusion. Before this, they received either no intervention (control group) or one episode of 5 min of ischemia, followed by 5 min of reperfusion (PC group), or an intravenous administration of the sphingomyelinase inhibitor D609. Myocardial content of ceramide and DAG was measured using the DAG kinase assay at different time points of the experiment. Apoptosis was detected and quantified by a sandwich enzyme immunoassay. Both AR and infarct size were measured using blue dye injection and triphenyltetrazolium chloride staining. Control hearts exhibited a peak of ceramide production at 5 min of the prolonged ischemia, with a mean value averaging 64 ± 5 ng/mg tissue (P < 0.05 vs. 48 ± 4 ng/mg at baseline). In contrast, ischemic PC and D609 prevented ceramide increase during the prolonged ischemia. Myocardial DAG content was increased only in PC hearts at 30 min of ischemia. Preconditioned and D609 groups developed less apoptosis, as well as a limited infarct size, compared with the control group. These results suggest that the antiapoptotic effect of PC may be due to a reduced ceramide production during sustained ischemia in the rabbit heart.

ischemia; reperfusion; apoptosis; necrosis

myocardial cell death after ischemia-reperfusion occurs by two different mechanisms, i.e., necrosis and apoptosis (7). Although the mechanisms of necrosis and apoptosis differ, it has been clearly demonstrated that ischemic preconditioning can attenuate both phenomena (29). Ceramide is a secondary messenger that can mediate apoptosis triggered via activation of CD95 (APO-1/Fas) and tumor necrosis factor receptors (4, 15). Indeed, apoptosis is often preceded by the accumulation of ceramide, and cell-permeable ceramide analogs induce apoptosis in several cell types (24). Ceramide is generated primarily from sphingomyelin by activated sphingomyelinases, although the increase of de novo synthesis of ceramide has also been reported (3, 11). Bielawska et al. (2) showed, using both in vitro and in vivo rat models, that the ceramide signaling pathway can be involved in ischemia-reperfusion-induced death of cardiomyocytes. In different cell types, sn-1,2-diacylglycerol (DAG), the natural activator of protein kinase C (PKC), which is involved in ischemic preconditioning, can inhibit the sphingomyelin-ceramide apoptotic pathway (13, 25, 34). The primary objective of the present study was to determine whether the antiapoptotic effect of preconditioning might be related to a reduced ceramide production during sustained ischemia in the in vivo rabbit heart model. A secondary goal of the study was to investigate whether altered production of DAG, which has been reported to inhibit the sphingomyelin-ceramide pathway, might explain the antiapoptotic effect of preconditioning.

EXPERIMENTAL PROCEDURES

All animals were treated in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

Surgical Preparation

Male New Zealand White rabbits (2.2–2.5 kg wt) were anesthetized by intramuscular injections of xylazine (5 mg/kg) and ketamine (50 mg/kg), as previously described (9). A continuous intravenous infusion of a mixture of xylazine (20–50 µg·kg−1·min−1) and ketamine (40–100 µg·kg−1·min−1) was maintained throughout the experiment. After a midline cervical incision was made, a tracheotomy was performed, and the animals were ventilated with room air. A cannula was inserted into the right internal jugular vein for administration of drugs and fluids, and into the left carotid artery for measurement of blood pressure. After an intravenous bolus administration of fentanyl (10 mg/kg), a left thoracotomy was performed in the fourth left intercostal space. The pericardium was opened and the heart exposed. A 3-0 silk suture attached to a small curved needle was passed around a marginal branch of the left circumflex coronary artery. Both ends of the thread were passed through a small vinyl tube to form a snare that could be tightened to occlude and loosened to reperfuse the artery. Body temperature was monitored via an intraperitoneal thermometer and kept constant by means of a heating pad. Heart rate and arterial pressure were monitored continuously throughout the experiment on a recorder (Gould; Cleveland, OH). After the surgical procedure, a 15-min hemodynamic stabilization period was observed.

Protocol 1: Myocardial Ceramide and DAG

Experimental design. All animals were prepared to undergo a 30-min sustained coronary occlusion (Fig. 1). Before undergoing prolonged ischemia, the animals were subjected to a 10-min treatment
Preparation of lipid extracts. Frozen samples of myocardium were powdered in a mortar under liquid nitrogen and lipids were subsequently extracted according to Folch et al. (5). Briefly, myocardial tissue was homogenized with a 2/1 chloroform/methanol mixture (by volume) to a final 20-fold volume dilution of the tissue sample. After 2 h at 4°C, 4.2 ml/g of KCl 0.37 M were added to the homogenate. The organic and aqueous phases were separated by centrifugation (8 min, 3,000 g). The organic phase was removed and evaporated to dryness. The aqueous phase was reextracted by chloroform. Dried lipid extracts were stored at −20°C under nitrogen until analysis.

Ceramide and DAG assay. Ceramide and DAG contents were quantified using the DAG kinase assay as the amount of 32P incorporated on phosphorylation of ceramide and DAG to ceramide 1-phosphate and phosphatidic acid, respectively, by DAG kinase from Escherichia coli (Biomol; Plymouth Meeting, PA). Briefly, [γ-32P]ATP (10 mM, 10 μCi) was incubated with the enzyme and lipid samples in an imidazole buffer (pH 6.60), as described by Preiss et al. (30). Ceramide 1-phosphate and phosphatidic acid were resolved by thin-layer chromatography (TLC) with the use of CHCl3/CH3OH/CH3COOH (65:15:5, vol/vol/vol) as a solvent. Ceramide 1-phosphate and phosphatidic acid were, respectively, identified by autoradiography at retention factor (RF) 0.25 and RF 0.45, and quantified with the use of scintillation counting. The levels of ceramide and DAG were quantified by comparison to a concomitantly run standard curve comprising known amounts of ceramide and diacylglycerol (Sigma; St. Louis, MO) and normalized to [3H]triglyceride introduced as an external standard during lipid extraction.

Protocol 2: Myocardial Apoptosis

Experimental design. Twenty-two additional rabbits were used in protocol 2. They were randomly assigned in one of three groups: control (n = 8), PC (n = 8), or D609 (n = 6) (Fig. 1). At the end of the 4-h reperfusion period, myocardial samples were carefully harvested from the previously ischemic and nonischemic areas for further assessment of apoptosis with the use of the sandwich enzyme immunoassay.

Sandwich enzyme immunoassay. For quantification of DNA fragmentation, specific determination of cytosolic mononucleosomes and oligonucleosomes was performed using an ELISA kit (Boehringer Mannheim), designed to quantify cytosolic oligonucleosome-bound DNA (18, 28, 29). This assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively (17).

Fifty milligrams of transmural samples from nonischemic and ischemic areas were isolated, washed of blood, disintegrated in a tissue grinder, and incubated for 30 min at room temperature in a 800-μl lysis buffer supplied with the kit. The homogenate was centrifuged at 13,000 g for 30 min. The supernatant (i.e., cytosolic fraction) was used directly as the antigen source in the sandwich ELISA and incubated for 2 h at room temperature with the immunoreagent mix included in the kit. Incubation buffer (instead of the sample solution) and DNA-histone complex included in the kit were used as background and positive control, respectively. Three values from the absorbance measurements (405/490 nm) of the samples were averaged, and the background value of the immunoassay was subtracted from each of these mean values. The positive control was used as validity internal control of the technique.

Protocol 3: Myocardial Necrosis

Experimental design. Thirty-three rabbits were randomly assigned into one of three groups and underwent 30 min of coronary artery occlusion, followed by 4 h of reperfusion (Fig. 1). Before this ischemic insult, animals underwent either no intervention for 10 min (control group; n = 10), or preconditioning by 5 min of ischemia, followed by 5 min of reperfusion (PC group; n = 10), or an intravenous bolus injection of D609 (0.1 mg/kg), 10 min before the
sustained ischemia (D609 group; n = 13). In all three groups, hearts were excised at the end of the 4-h reperfusion period (Fig. 1).

AR and infarct size determination. At the end of the 4-h reperfusion period, the coronary artery was reocluded and 0.5 mg/kg Uniprise blue pigment was injected intravenously in the jugular vein to delineate the infarct region in vivo AR, as previously described (26). With this technique, the previously nonischemic myocardium appears blue, whereas the previously ischemic myocardium (AR) remains unstained. Anesthetized rabbits were then euthanized by an intravenous injection of 4 meq KCl. The heart was excised and cut into five or six 2-mm-thick transverse slices, parallel to the atrioventricular groove. After right ventricular tissue was removed, each heart slice was weighed. The basal surface of each slice was photographed for later measurement of the AR. Each slice was then incubated for 15 min in a 1% solution of triphenyltetrazolium chloride at 37°C to differentiate infarcted (pale) from viable (brick red) myocardial area (32). The slices were then rephotographed. Enlarged projections of these slices were traced for determination of the boundaries of the AR and the area of necrosis (AN). Extent of the AR and AN was quantified by computerized planimetry and corrected for the weight of the tissue slices. Total weights of the AR and the AN were then calculated and expressed in grams and as percentage of total left ventricle (LV), and percentage of weight of the AR and the AN were then calculated and expressed in grams and as percentage of total left ventricle (LV), and percentage of risk region decreased from viable (brick red) myocardial area (32). The slices were then incubated for 15 min in a 1% solution of triphenyltetrazolium chloride at 37°C to differentiate infarcted (pale) from viable (brick red) myocardial area (32). The slices were then rephotographed. Enlarged projections of these slices were traced for determination of the boundaries of the AR and the area of necrosis (AN). Extent of the AR and AN was quantified by computerized planimetry and corrected for the weight of the tissue slices. Total weights of the AR and the AN were then calculated and expressed in grams and as percentage of total left ventricle (LV), and percentage of

**Statistical Analysis**

Comparisons between groups were performed using two-factor ANOVA with replication for ceramide and DAG contents. Normalized absorbances were compared by one-factor ANOVA. Means were compared by the Fisher’s test when a significant F value was obtained. Differences in the relationship between infarct size and AR were evaluated by ANCOVA and post hoc Tukey’s test, with infarct size as the dependent variable and AR as the covariant. All values are expressed as means ± SE. Statistical significance was defined as a value of P < 0.05.

**RESULTS**

**Protocol 1: Ceramide and DAG Myocardial Content**

**Ceramide content.** Just before the sustained ischemic insult (i.e., after the preconditioning regimen), PC hearts exhibited a significantly reduced ceramide content that averaged 29.1 ± 1.9 ng/mg tissue (P < 0.01 vs. control).

Control hearts exhibited a significant increase in myocardial ceramide during the first minute of the prolonged coronary artery occlusion with mean ceramide level averaging 64.0 ± 4.5 ng/mg tissue (wet weight) at 5 min of ischemia versus 47.5 ± 3.5 ng/mg tissue at baseline (P < 0.01) (Fig. 2). However, this peak was transient because ceramide content returned to near-baseline values as soon as 10 min into the sustained ischemia. In the meantime, ceramide failed to significantly increase in both the PC and D609 groups and remained significantly lower than control values, averaging 36.3 ± 4.8 and 37.9 ± 3.1 ng/mg tissue at 5 min of ischemia in PC and D609 hearts [P < 0.01 vs. control, P = 0.05 vs. D609 hearts, exhibited a dramatic late increase in DAG content that averaged 85.4 ± 21.8 ng/mg tissue in preconditioned versus 36.9 ± 15.8 and 41.9 ± 6.0 ng/mg tissue in the control and D609 groups, respectively (P < 0.05 vs. control and D609).

**DAG content.** In the early minutes of the prolonged ischemic insult, DAG content did not significantly vary in any of the three groups. Yet, at 30 min of ischemia, preconditioned, but not D609 hearts, exhibited a dramatic late increase in DAG content that averaged 85.4 ± 21.8 ng/mg tissue in preconditioned versus 36.9 ± 15.8 and 41.9 ± 6.0 ng/mg tissue in the control and D609 groups, respectively (P < 0.05 vs. control and D609).

**Protocol 2: Myocardial Apoptosis**

After 30 min of coronary artery occlusion and 4 h of reperfusion, DNA fragmentation in nonischemic regions was very low and not significantly different among control, preconditioned and D609 hearts, with normalized absorbances averaging 0.21 ± 0.09, 0.22 ± 0.17, and 0.28 ± 0.09, respectively (Fig. 3). As expected, DNA fragmentation was much higher in ischemic than in nonischemic regions. In ischemic regions, preconditioned and D609-treated hearts exhibited significantly less DNA fragmentation than controls, with mean normalized absorbance averaging 0.71 ± 0.15 and 0.68 ± 0.33 versus 1 for ischemic controls (P < 0.001 vs. control for both groups) (Fig. 3).
Protocol 3: Myocardial Necrosis

Heart rate and mean blood pressure were not significantly different among the three groups of animals. AR was comparable among the three groups, averaging 26.5 ± 3.1%, 28.5 ± 2.8%, and 24.8 ± 1.9% of LV weight in control, PC, and D609 groups, respectively (P = NS among the three groups). For control animals, infarct size averaged 56.4 ± 9.1% of the risk region. Preconditioning significantly reduced infarct size to 14.5 ± 3.4% of the risk region (P < 0.05). Similarly, although to a lesser extent, D609 pretreatment reduced infarct size to 33.9 ± 6.1% of the AR (P < 0.05 vs. control group, P = NS vs. PC) (Fig. 4).

DISCUSSION

In the present study, we demonstrated that ischemic preconditioning limits both myocardial apoptosis and necrosis in the in vivo rabbit heart. This protective effect was accompanied by a reduction in myocardial ceramide content during the sustained ischemia. Reduced ceramide production after blockade of sphingomyelinase by D609 resulted in an antiapoptotic effect comparable to that of preconditioning.

Gottlieb et al. (8) first demonstrated that preconditioning can limit apoptosis in the isolated rabbit cardiomyocyte preparation. Piot et al. (29) reported an antiapoptotic effect of preconditioning using the in vivo rat model of myocardial infarction. In the present study, we extended this demonstration by using the classic rabbit heart model of ischemic preconditioning. ELISA data showed a clear antiapoptotic effect of preconditioning.

Several hypotheses have been put forward to try to explain the antiapoptotic effect of preconditioning. Maulik et al. (20) suggested that this protective effect was related to the upregulation of the antideath gene bcl-2. Nakamura et al. (22) reported that preconditioning can decrease expression of the proapoptotic protein Bax in the rat heart. Piot et al. (28) suggested that ischemic preconditioning attenuates apoptosis in in vivo rat hearts via the prevention of caspase 1 and 3 activation. Similar results have been reported by Yadav et al. (33) regarding protection of the mouse liver against ischemia-induced apoptosis. Recent evidence indicates that the sphingomyelin-ceramide signaling pathway is involved in apoptosis. Ceramide can induce apoptosis via two independent pathways, i.e., the JNK cascade and transcriptional regulation of gene products like Fas ligand or tumor necrosis factor-α, or directly through mitochondrial permeability transition (MPT). Several recent reports indirectly support the latter possibility because 1) MPT is known to play an important role in apoptosis, 2) MPT is likely involved in ischemic preconditioning (12, 14), and 3) ceramide can modulate MPT (27). Yet, further studies are needed to confirm this hypothesis. Bielawska et al. (2) reported, in rat neonatal cardiomyocyte culture, that ceramide level dramatically increases during simulated ischemia. Our in vivo results regarding ceramide production in the control group are in close agreement with those of Bielawska et al. (2) as opposed to Murase et al. (21), who found no change in ceramide content after 5 min of global ischemia-reperfusion in the isolated rat heart. This apparent discrepancy with our data is possibly due to the use of different experimental models and techniques for the measurement of myocardial ceramide. Le-cour et al. (16) found that perfusion of C2-ceramide limits infarct size in the isolated rat heart. Myocardial ceramide content was not measured in that study and it is unclear how ceramide, which mediates apoptosis and inhibits the PKC pathway, can mimic ischemic preconditioning. The rapid enhancement of ceramide production we observed after a brief period of ischemia is consistent with previous reports indicating that neutral and acid sphingomyelinases are rapidly and transiently activated by diverse exogenous stimuli (19). Conversely, Zhang et al. (35) recently reported that myocardial ceramide accumulation after ischemia-reperfusion might be related to a reduced degradation via ceramidases. In the present study, we found that ceramide production is significantly reduced during the early minutes of the sustained occlusion in preconditioned hearts. Beresewicz et al. (1) recently reported an enhanced myocardial ceramide accumulation during reperfusion after a prolonged ischemic insult in the rat model. Interestingly, this ceramide accumulation, which was blunted by preconditioning, already existed during the ischemic period and reperfusion only exaggerated this phenomenon (1). On purpose, we did not assess myocardial ceramide content during the final reperfusion for two reasons. First, although it is clear that after an ischemic insult, apoptotic death mostly occurs during reperfusion, the initial triggers of apoptosis act as soon as the early minutes of ischemia, and preconditioning has clearly been shown to become active at that time. Second, any difference in myocardial ceramide content during reperfusion may partly reflect the expected larger amount of dead myocardium in control than in preconditioned hearts. This latter limitation also stands for interpretation of DAG and ceramide data at 30 min of ischemia. One cannot rule out, however, that part of the antiapoptotic effect of preconditioning might also be related to any event that occurred during reperfusion, because we did not assess myocardial ceramide at that time.

For the first time, we report that reduced ceramide accumulation was associated with a limited apoptotic death in PC hearts. To further investigate whether limited apoptosis was related to the reduced ceramide production, we used the methylxanthate D609, which has been reported to inhibit activation of sphingomyelinases that hydrolyze sphingomyelin into ceramide. Sphingomyelinases are rapidly and transiently activated by diverse exogenous stimuli, leading to increases in ceramide levels in a time frame of seconds to minutes (19). Ceramide can also be synthesized de novo by a ceramide synthase, but this pathway is likely not involved in our experimental condi-
tions because it requires several hours to generate detectable ceramide (3). In the present study, preventing ceramide increase with D609 resulted in a significant reduction in apoptosis after ischemia-reperfusion. This indirectly suggests that the antiapoptotic effect of preconditioning may be due to its ability to limit myocardial ceramide content. The clear antiapoptotic effect of D609 was accompanied by a slight but significant reduction of infarct size, although to a lesser extent than ischemic preconditioning. Some reports indicate that D609 cant reduction of infarct size, although to a lesser extent than ischemic preconditioning. Some reports indicate that D609 also inhibits phosphatidylyceroline-specific phospholipase C, and as a result, can attenuate the activation/translocation of one or several PKC isofoms (6, 31). However, protection induced by ischemic preconditioning is related to activation, but not inhibition, of the DAG-PKC pathway. Also, D609 had no significant effect on DAG content at 30 min of ischemia in the present study, as well as at 5 and 10 min of ischemia (data not shown). Although we cannot rule out that D609 acted on phosphatidylyceroline-phospholipase C, we believe that most of its effect observed in the present study is related to an action on the sphingomyelin-ceramide pathway.

The mechanism of this blunted production of ceramide by preconditioning in the early minutes of the test ischemic episode is unknown. Because it has been reported that DAG can inhibit the sphingomyelin-ceramide pathway, we assessed the time course of myocardial DAG production in preconditioned hearts (13). We found a dramatic but late increase in DAG content in preconditioned hearts, with mean myocardial content being twice that of control hearts at the end of the prolonged ischemic episode. A biphasic activation of the PKC pathway has been reported in vitro models, with the second peak being more prolonged and much higher than the early one (23). However, in the present study, this enhanced DAG production in preconditioned hearts occurred several minutes after the ceramide peak, strongly suggesting that the early limitation of ceramide production in preconditioned hearts is likely not due to the late increased DAG accumulation. However, because we did not measure DAG before 5 min of ischemia, we cannot fully rule out that we missed an earlier peak of DAG production, as suggested by a previous study from our group (10). In this case, one may hypothesize that activation of the phospholipase C-DAG-PKC pathway would be responsible for the limited ceramide production. This does not exclude any antiapoptotic effect of the enhanced and delayed DAG production, which might act on other targets of this pathway. This enhanced late DAG production in preconditioned versus D609 hearts may also account for the larger infarct size reduction in the former group.

Overall, our results suggest that reduction of ceramide during ischemia may play a role (although not exclusive) in the antiapoptotic effect of preconditioning. Further investigations are required to fully understand the mechanism of this reduced ceramide production.

REFERENCES


