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ACCUMULATION OF SPECIFIC CERAMIDES IN ISCHEMIC/REPERFUSED RAT HEART; EFFECT OF ISCHEMIC PRECONDITIONING

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Ceramide signalling has been implicated in the mechanism of myocardial ischemia/reperfusion injury (IR). This study tested the hypothesis that ceramides containing a specific amino-linked acyl residue mediate the injury, and that ischemic preconditioning (IPC) affords myocardial protection because it prevents increased ceramide accumulation in IR myocardium. Perfused rat hearts were subjected either to the sham perfusion or to 30 min global ischemia, 30 min ischemia/30 min reperfusion (IR) or were preconditioned prior to the standard IR. The ventricles were harvested for biochemical assay that involved transmethylation of ceramide amino-linked acyl residues, and gas liquid chromatography measurement of acyl methyl esters. Fourteen ceramides containing myrystic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidic, arachidonic, eicosapentaenoic, behenic, docosapentaenoic, docosahexaenoic or nervonic acid were identified in the myocardium of rats. The total basal ceramide concentration in the myocardium was 135 nmol/g tissue, and it was increased by 14.1% and 48.4% in the ischemia and IR group, respectively. However, in fact, IR increased the accumulation of only 7 out of 14 ceramides identified in the heart (i.e., those containing palmitic, stearic, oleic, linoleic, and arachidonic acid), and the relative magnitude of these increases varied between the particular ceramides and was independent from their basal tissue concentration. IPC improved postischemic hemodynamic recovery and partially prevented the reperfusion-induced increases in these 7 ceramides, while the other ceramides were unaffected by IPC. These results support the role of the specific ceramide signalling in the mechanism of myocardial IR injury. We speculate that by preventing tissue accumulation of certain ceramides, IPC attenuates this signalling, that adds to the mechanism of myocardial protection afforded by IPC.

Key words: *Ceramide; Ischemia/reperfusion; Ischemia/reperfusion injury; Ischemic preconditioning; Isolated rat heart;*

INTRODUCTION

In addition to their role in cell structure, sphingolipids have a role in signal transduction and cell regulation. Thus, a large number of agonists and stress signals have been demonstrated to induce the hydrolysis of sphingomyelin, with resulting accumulation of ceramide and other bioactive molecules, serving as second messengers for various cellular processes (1-4).

Several lines of evidence suggest a role of sphingolipid signalling cascade in the mechanism of tissue ischemia/reperfusion (IR) injury, including myocardial injury. First, changes in ceramide metabolism have been associated with tissue damage during IR in rat brain (5), mice kidney (6), and rat heart (7-9). For example, in the rat heart coronary occlusion model, the total ceramide content in the ischemic myocardium has been found to increase after 30 min of ischemia, and reperfusion exaggerated this effect (7). Second, it has been demonstrated that IR increases tissue cytokines such as TNF-α and Il-1β in the myocardium, that these cytokines may contribute to the myocardial injury, in particular to myocyte apoptosis (10-12), and that ceramide mediates the actions of TNF-α and other cytokines in cell injury (2). The role of sphingolipid cascade in the TNF-α-induced apoptosis has been demonstrated also in cardiomyocytes (13). Third, IR, and particularly reperfusion, is known to trigger myocardial apoptosis (14-16) and it has been suggested that ceramide serves as an intermediate signal to initiate apoptosis in rat heart subjected to IR (7), and in neonatal rat cardiomiocytes during hypoxia/reoxygenation (17). Fourth, ceramide has been shown to reduce endothelium-dependent vasodilation and to increase superoxide production in coronary arteries (18), which might contribute to the pathophysiology of IR.

It has been demonstrated that the induction of apoptosis in leukaemic cells is due to the engagement of specific pools of sphingomielin (19). Furthermore, we have demonstrated that prolonged exercise differentially reduces the tissue concentration of specific ceramides identified in rat skeletal muscle (20). The role of specific ceramides in myocardial IR has not been studied. Thus the first aim of the study was to verify that IR affects metabolism of only specific ceramides, (i.e., those containing a specific amino-linked fatty acid residues) and determine if they play a role in the mechanism of IR injury.

The second aim of the study was to examine the effect of ischemic preconditioning (IPC) on IR-induced tissue accumulation of various ceramides present in the heart. IPC is a phenomenon in which brief periods of ischemia and reperfusion render the heart very resistant to a subsequent more sustained ischemic insult (21). Although IPC is a most potent means of protecting myocardium from IR injury, its molecular mechanism is not completely understood (22, 23). IPC has been demonstrated to attenuate over-production of TNF-α in IR heart (24) as well as various forms of myocardial IR injury, including myocyte apoptosis (25-27). Accordingly, we hypothesize that if some specific ceramides serve a role of the intermediate signal between stress signals

and myocardial injury in IR, the over-production of these ceramides and the IR injury should be attenuated by IPC.

MATERIALS AND METHODS

Isolated heart preparation

Male Wistar rats (260-350 g) were injected with 500 units of heparin sulfate, i.p., 20 min before being anaesthetised with penthobarbital sodium (50 mg/kg, i.p.). Hearts were excised and perfused in the Langendorff mode, at perfusion pressure of 70 mmHg, with prefiltered (5.0 μm Millipore filter) perfusion fluid containing, in mmol/l: 118 NaCl; 23.8 $NaHCO_3$; 4.7 KCl; 1.2 KH_2PO_4; 2.5 $CaCl_2$; 1.2 $MgSO_4$, and 11 glucose, and gassed with 95% O_2 + 5% CO_2 gas mixture giving pH 7.4 and pO_2 580-640 mmHg at 37°C. The left ventricular developed pressure (LVDP, peak systolic pressure minus end-diastolic pressure), left ventricular end-diastolic pressure (LVEDP), and heart rate were recorded via a fluid-filled latex balloon inserted into the left ventricle and connected to a pressure transducer (P23 Pressure Transducer, Gould Statham Instruments Inc.) and a recorder (Elema Shoenander Mingograph-81 polygraph, Stockholm, Sweden). The volume of the balloon was adjusted to obtain LVEDP of ~5 mmHg during the equilibration perfusion and it was not changed thereafter. The hearts were enclosed in a small, water-jacketed chamber. The temperature of the perfusate was thermostatically controlled and checked at regular intervals to ensure 37°C. The hearts were not stimulated. Global ischemia was induced by clamping the aortic inflow line and simultaneous immersing the heart in a small volume of the venous effluent. The immersion was stopped when the cannula was unclamped to achieve reperfusion. Coronary flow was quantified by a timed collection and weighing of perfusate exiting the right heart.

The study was approved by institutional Ethics Committee.

Experimental protocols

Within each of five types of experiments performed, all the hearts had an initial 30 min equilibration perfusion and then they were either:

(i) harvested for ceramide measurement (*Sham 30'*) or were subjected to
(ii) a further 90 min aerobic perfusion (*Sham 120'*);
(iii) a 30 min aerobic perfusion followed by 30 min global ischemia without reperfusion (*Ischemia*);
(iv) a 30 min aerobic perfusion + 30 min global ischemia + 30 min of reperfusion (*IR*);
(v) ischemic preconditioning stimulus consisting of three cycles of 5 min global ischemia/5 min reperfusion applied prior to the standard IR (*IPC*).

After the completion of each perfusion protocol, the hearts were freeze-clamped, and the ventricles were stored at -72°C for the further biochemical assay.

Assay of ceramide-fatty acid composition

The procedure involved tissue pulverization, ceramide separation, transmethylation of ceramide amino-linked acyl residues, and gas liquid chromatography identification and measurement of acyl methyl esters, as described before (20). Briefly, the frozen myocardial samples were pulverised in an aluminium mortar with the stainless pestle pre-cooled in liquid nitrogen. The powder was transferred to tubes containing methanol at temperature -20°C. Methanol contained butylated hydroxytoluene (Sigma), 30mg/100ml, as an antioxidant. The tubes were warmed to the room

temperature and chloroform was added. The chloroform phase was evaporated and the residue was dissolved in chloroform/methanol and was spotted on the thin-layer chromatoghraphy silica plates (Kieselgel 60, 0.22mm, Merck). The plates were first developed to one third of the plate's length with chloroform/methanol/25% NH_3 (20/5/0.2 v/v/v) (28). Next, they were dried and rechromatographied in heptane/isopropyl ether/acetic acid (60/40/3 v/v/v). The standard of ceramide (Sigma) was run along with the samples. The plates were dried at room temperature and sprayed with 0.5% solution of 3'7'-dichlorofluoresceine in absolute methanol. The lipid bands were visualised under UV light. The bands containing ceramide were scraped off the plates and transmethylated along with the gel using 14% boron fluoride in methanol at 100°C for 90 min (29). Methylpentadecanoic acid (Sigma) was added as an internal standard. The fatty acid methyl esters were identified and quantified by means of gas liquid chromatography using Hewlett-Packard 5890 Series II and a fused Hp-INNOWax capillary column (50 m). The area corresponding to the internal standards was used to calculate the content of the individual fatty acid methyl esters. The standards were purchased from Sigma

Statistics

All data are expressed as mean ± S.E.M. Significant inter-group differences were calculated using either Kruskal-Wallis test followed by Mann-Whitney's post hoc test (biochemical data) or one-way analysis of variance followed by Dunnet's post hoc test (functional data). Differences between groups were considered significant if the P value was < 0.05.

RESULTS

Ceramide fatty acid composition and ceramide concentration in sham perfused hearts

There were no significant differences in the ceramide fatty acid composition and total tissue ceramide concentration between the hearts subjected to 30 min and 120 min of aerobic sham perfusion (not shown). Consequently, these two sham groups were pooled and treated as one group in further statistical comparisons (pooled sham in *Table 1*).

As listed in Table 1, 14 ceramides, differing in their amino-linked fatty acid residue, were identified in sham-perfused hearts. The total myocardial concentration of ceramide was 135.1±11.5 nmol/g tissue. Five ceramides containing a saturated fatty acid residue (mostly palmitate and stearate) and 9 ceramides containing an unsaturated fatty acid contributed to 73% and 27% of the total ceramide concentration, respectively.

Effect of ischemia, IR, and IPC on myocardial ceramide

As shown in Table 1, 30 min of ischemia led to a 14.1% increase in the total myocardial ceramide concentration ($p < 0.05$). The total ceramide was elevated even further (by 48.4%, $p < 0.05$ vs. ischemia) by reperfusion, and this reperfusion-induced effect was partially prevented by IPC. The latter is evidenced by the fact that the total ceramide concentration was significantly

Table 1. Concentration of 14 different ceramides identified in rat heart as affected by ischemia alone, ischemia and reperfusion, and ischemic preconditioning

Ceramide Fatty Acid Residue	Pooled sham (n=11) (nmol/g)	Pooled sham (n=11) (% of Total)	Ischemia (n=5) (nmol/g)	IR (n=6) (nmol/g)	IPC (n=5) (nmol/g)
Myristic (14:0)	3.25 ± 0.3	2.4 ± 0.2	3.28 ± 0.4	3.63 ± 0.6	3.13 ± 0.8
Palmitic (16:0)	53.75 ± 6.4	39.8 ± 4.7	60.66 ± 3.3[a]	79.98 ± 6.1[a, b]	62.02 ± 6.2[a, c]
Palmitoleic (16:1)	7.01 ± 0.6	5.2 ± 0.4	6.21 ± 0.9	6.85 ± 1.7	5.76 ± 0.7
Stearic (18:0)	31.03 ± 5.5	23.0 ± 4.0	38.64 ± 6.8[a]	47.86 ± 6.0[a, b]	41.49 ± 8.4[a, c]
Oleic (18:1)	6.84 ± 0.9	5.1 ± 0.7	8.18 ± 0.9[a]	10.78 ± 1.2[a, b]	10.24 ± 2.0[a, b]
Linoleic (18:2)	4.39 ± 0.9	3.2 ± 0.7	9.45 ± 1.2[a]	16.74 ± 2.7[a, b]	13.18 ± 2.2[a, b, c]
Linolenic (18:3)	1.51 ± 0.8	1.1 ± 0.6	1.40 ± 0.3	1.76 ± 0.5	1.48 ± 0.2
Arachidic (20:0)	5.91 ± 0.7	4.4 ± 0.7	5.02 ± 0.7	5.48 ± 1.5	5.29 ± 0.3
Arachidonic (20:4)	4.02 ± 0.4	3.0 ± 1.0	5.00 ± 0.4[a]	8.22 ± 0.6[a, b]	5.43 ± 0.9[a, c]
Eicosapentaenic (20:5)	5.53 ± 1.2	4.1 ± 0.9	5.17 ± 0.6	5.48 ± 0.7	5.73 ± 0.9
Behenic (22:0)	5.01 ± 0.7	3.7 ± 0.7	4.01 ± 0.6	4.46 ± 0.9	4.21 ± 0.9
Docosapentaenoic (22:5)	1.43 ± 0.4	1.1 ± 0.3	1.29 ± 0.3	2.35 ± 0.6[a, b]	1.39 ± 0.2[c]
Docosaheksaenic (22:6)	0.93 ± 0.2	0.7 ± 0.1	1.09 ± 0.2	1.11 ± 0.1[a]	1.17 ± 0.2[a]
Nervonic (24:1)	4.52 ± 0.7	3.3 ± 0.5	4.78 ± 1.1	5.90 ± 1.5	5.55 ± 1.6
TS	98.95 ± 10.7	73.2 ± 7.8	111.66 ± 9.4[a]	141.40 ± 12.1[a, b]	116.14 ± 14.5[a, c]
TUN	36.18 ± 2.7	26.7 ± 2.0	42.59 ± 2.3	59.19 ± 3.8[a, b]	49.93 ± 1.1[a, c]
Total	135.13 ± 11.3	100.0 ± 0.0	154.25 ± 11.9[a]	200.60 ± 12.8[a, b]	166.07 ± 14.9[a, b, c]

Values are means ± S.D. of n experiments/group; After 30 min stabilization perfusion, the hearts were either: (i) immediately harvested or subjected to a further 90 min aerobic perfusion (pooled sham) or were subjected to (ii) 30 min global ischemia (ischemia); (iii) 30 min global ischemia + 30 min reperfusion (IR) or (iv) the ischemic preconditioning prior to the standard IR (IPC).
TS, total content of the saturated fatty acids; TUN, total content of the unsaturated fatty acids. Total- the sum of all ceramide-fatty acids
[a] $p < 0.05$ vs. Sham; [b] $p < 0.05$ vs. Ischemia; [c] $p < 0.05$ vs. IR

smaller in IPC versus IR group, and it was significantly greater in IPC versus ischemia group.

IR increased accumulation of only 7 out of 14 ceramides identified in the heart, and it was only these changes that accounted for the increase in the total myocardial ceramide concentration. As shown in (*Fig. 1*), ischemia alone caused a varied increases (by 13-115%) in the myocardial level of ceramides containing palmitic, stearic, oleic, linoleic or arachidonic acid, the per cent increase in the ceramide containing linoleic acid (by 115%) being the biggest. The levels of these 5 ceramides were approximately tripled (increases by 48-281%) by reperfusion. In addition, although ischemia had no significant effect on ceramides containing

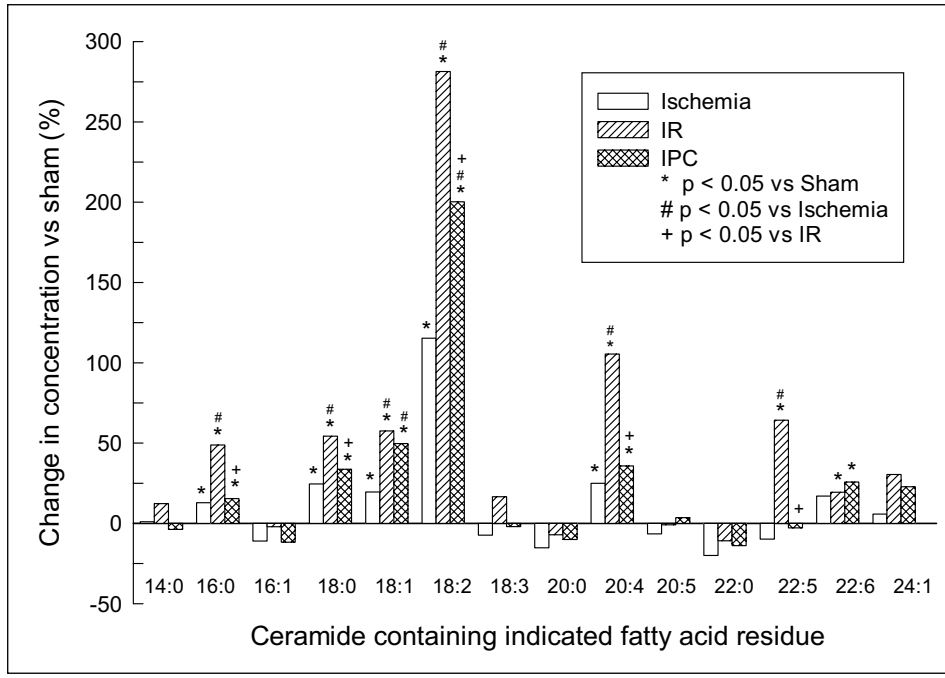

Fig. 1. Relative changes in the myocardial concentration of 14 ceramides containing indicated amino-linked fatty acid residues caused by ischemia, ischemia + reperfusion (IR), and ischemic preconditioning (IPC) in isolated rat heart. Percent changes from respective sham values (denoted as 0 line) are plotted. Each point represents mean from 5-6 measurements. SEMs are omitted to simplify the plots.

docosapentaenoic and docosaheksaenoic acid, their levels were significantly increased by reperfusion. Of note, the relative magnitude of IR-induced myocardial accumulation of the 7 ceramides was not proportional to their basal tissue concentrations. For example, although ceramides containing palmitic and stearic acid residue are most abundant in rat heart (40% and 23% of total myocardial concentration, respectively), ischemia increased their concentration by only 13-25%, and IR by 48-54%. However, the level of ceramide containing linoleic acid was increased by 115% and 281%, respectively, although this ceramide contributes to only 3.2% of the total myocardial ceramide. Similarly, ceramide containing arachidonic acid contributes to 3% of the total ceramide and reperfusion increased its concentration by 105%.

The reperfusion-induced effects in the myocardial ceramides were differentially affected by IPC. They were completely prevented in ceramides containing palmitic, stearic, arachidonic and docosapentaenoic acid, partially prevented in ceramides containing oleic and linoleic acid, and not affected in ceramide containing docosaheksaenoic acid.

Effect of IPC on post-ischemic functional recovery

As exemplified in (*Fig. 2*), there were no significant differences in baseline values (i.e., those obtained after 30 min equilibration perfusion) for coronary flow, LVDP, and LVEDP between any of the experimental groups studied. Moreover, there were no significant differences between baseline values and those obtained at the conclusion of the perfusion protocol, for coronary flow (14.9 ± 0.4 ml/min vs. 14.6 ±.0.7 ml/min, respectively) and LVDP (113 ± 8 mmHg vs. 109 ± 10 mmHg, respectively) in the hearts subjected to 120 min sham perfusion, confirming a stability of our heart preparation.

As illustrated in (*Fig. 2*), the time-course of the post-ischemic recovery of coronary flow and LVDP was faster, and the final recoveries were significantly more complete in IPC vs IR group. Thus, the percent recoveries of coronary flow and LVDP amounted to 102% and 76% in IPC group, respectively, and only to 61% and 22% in IR group, respectively.

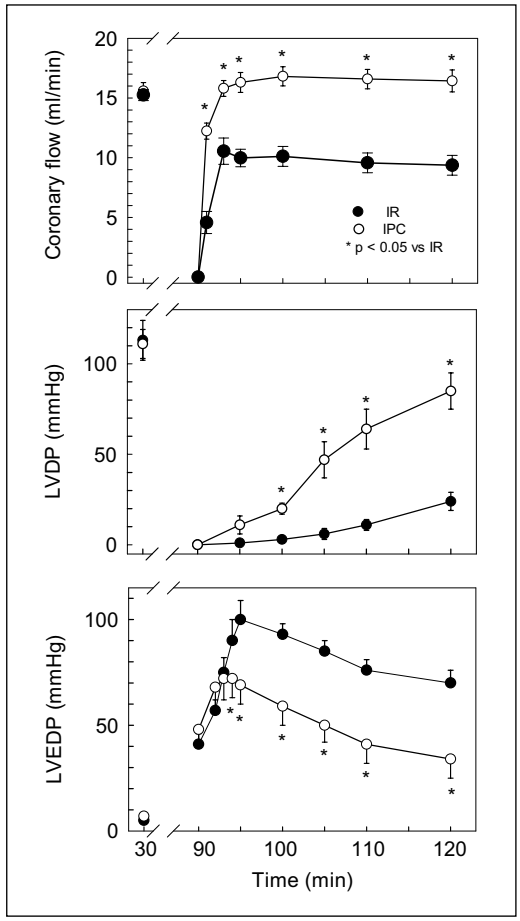

Fig. 2. Effect of ischemic preconditioning on the time-course and the post-ischemic recovery of coronary flow and left ventricular developed tension (LVDP), and on reperfusion-induced changes in left ventricular end diastolic pressure (LVEDP) in isolated rat heart. After 30 min equilibration, the hearts were subjected either to 30 min global ischemia + 30 min reperfusion (IR) or to ischemic preconditioning prior to IR (IPC). Each point represents mean ± S.E.M. for 5-6 hearts.

In IR as well as in IPC group, 30 min ischemia caused significant increase in LVEDP, denoting the development of a myocardial contracture. As shown in Fig. 2 (bottom panel), the contracture in both these experimental groups was similar at 30 min of ischemia (48 ± 7 mmHg and 41 ± 5 mmHg, respectively, $p > 0.05$), it further increased during the first 3-5 min of the reperfusion, and it continued to fall slowly thereafter. Importantly, the peak reperfusion contracture and the contracture at the end of 30 min reperfusion were significantly smaller in IPC versus IR group.

DISCUSSION

The main findings of this study were as follows: (i) we have analysed, for the first time, the fatty acid composition of myocardial ceramide, and identified 14 ceramides, containing different fatty acid residues, in perfused rat heart; (ii) ischemia caused modest increase in the total myocardial ceramide concentration, and reperfusion exaggerated this effect further; (iii) we demonstrate, for the first time, that IR caused varied increases in the myocardial concentration of only 7 out of 14 myocardial ceramides, and that IPC prevented or attenuated this effect along with affording myocardial protection. These results support the role of specific ceramide signalling in the mechanism of post-ischemic myocardial injury. We speculate that by preventing myocardial accumulation of certain ceramides, IPC attenuates this signalling, that adds to the mechanism of myocardial protection afforded by IPC.

Myocardial ceramide and its modification by IR

To address the role of specific ceramides in the mechanism of myocardial IR injury, we have analysed the fatty acid composition of myocardial ceramide at baseline and during ischemia and reperfusion. The baseline measurements, when confronted with our earlier measurements performed in rat skeletal muscle (20), reveal a number of tissue-specific differences in ceramide composition, which may be of a functional consequence. Thus, 14 and 12 ceramides, differing in their amino-linked acyl residue, were identified in rat myocardium and skeletal muscle, respectively, with the ceramides containing arachidic and docosapentaenoic acid residue not identified in the skeletal muscle. In both muscles, ceramides containing saturated fatty acid contributed to nearly 75% of the total ceramide content, and those containing palmitic and stearic acid were most abundant. While oleic acid was the most abundant ceramide-unsaturated fatty acid in the skeletal muscle, in the heart, the 9 identified unsaturated fatty acids were distributed more evenly among ceramide moieties.

The present study demonstrated that 30 min global ischemia modestly increases the total myocardial ceramide concentration, and that reperfusion greatly exaggerates this effect, in isolated rat heart. Two previous studies have reported qualitatively similar result in a model of 30 min ischemia and reperfusion in the

isolated and *in vivo* rat heart (7, 8). Likewise, ceramide has been shown to accumulate in the primary cultures of neonatal rat cardiomyocytes during hypoxia and reoxygenation (17). However, no change in myocardial ceramide was noted in isolated rat heart subjected to 5 min ischemia and 5 min reperfusion (30). Finally, in the study of Zhang et al., 30 min ischemia followed by reperfusion, but not ischemia alone, significantly increased the level of ceramide in *in vivo* rat heart (9). Altogether these results suggest that ischemia increases myocardial ceramide concentration only when it is severe enough, and that reperfusion is a particularly strong stimulus for ceramide to accumulate in the myocardium.

An intriguing new finding of the present study is that IR resulted in the increased accumulation of only 7 out of 14 ceramides identified in rat heart, that the magnitude of these increases varied between particular ceramides, and that it was independent from their basal tissue concentrations (cf. *Fig. 1*). These results support the hypothesis that during IR, at least some of myocardial ceramides undergo individual regulation of their tissue concentration, and therefore, that a specific ceramide signalling plays a role in the mechanism of IR injury in rat heart. However, to verify this hypothesis, a number of problems, which were not addressed in this study, need to be resolved.

First, it remains to be determined whether, indeed, various long chain ceramides present in the myocardium differ in their biological effects. Currently, the information on biological effects of only short chain cell permeable ceramides is available.

Second, it remains to be verified whether, indeed, the pattern of IR-induced changes in the concentrations of the individual ceramides shows a stress type-specificity. Actually, this concept finds support in our earlier finding that prolonged exercise differentially reduces the tissue concentration of various ceramides identified in rat skeletal muscle (20). As regards IR, the available evidence only suggests that myocardial metabolism of total ceramide is differentially regulated by IR vs. hypoxia/reoxygenation. Ceramide is produced primary by the hydrolysis of membrane sphingomyelin through a number of isoforms of sphingomyelinase, or from palmitoyl CoA and serine through *de novo* synthesis. Ceramide, once generated, can be metabolised or converted to other molecules by various enzymes, including ceramidase (2). The neutral and acidic isoforms of sphingomyelinase as well as ceramidase have been identified in rat heart (9). Actually, all these enzymes are regulated by cytokines in various cellular systems. Thus, the acidic and neutral shingomyelinases were found to be stimulated by TNF-α, Il-1β, and interferon-γ, and ceramidase can be activated or inhibited by these cytokines (31-33). In the cultured rat cardiac myocytes, hypoxia and reoxygenation has been reported to induce the activation of neutral sphingomyelinase and then the accumulation of ceramide (17) In contrast, in *in vivo* rat heart, ischemia followed by reperfusion was reported to decrease the activities of neutral and acidic sphingomyelinases and the accompanying accumulation of ceramide was attributed to decreased activity of ceramidase (9). It remains to be determined whether the differences between hypoxia/reoxygenation and IR in the regulation of the total tissue ceramide

concentration translate into differences in tissue accumulation of the individual ceramides.

Third, the molecular mechanism of the differential response of the individual ceramides to IR remains to be identified. Theoretically, the mechanism may involve a change in the activity of some substrate-specific isoforms of the enzymes involved in the ceramide metabolism as well as a site-specific regulation of the enzymes.

Effects of IPC

In our experimental model, the hearts subjected to IR developed prominent post-ischemic contracture and demonstrated impaired post-ischemic recovery of coronary flow and LVDP. These functional changes were attenuated by IPC, indicating that IR injury was attenuated in the hearts subjected to IPC. It remains uncertain if it was the attenuation of stunning, apoptosis or necrosis, which accounted for the beneficial effect of IPC. A new finding of this study is that, along with the functional protection, IPC prevented or attenuated reperfusion-induced increases in the myocardial concentration of only certain ceramides. These data are compatible with the hypothesis that there is a causal relationship between tissue injury and ceramide accumulation in rat heart subjected to IR, although it is not apparent from our study whether it was the altered ceramide metabolism, which mediated IR injury or rather *vice versa*.

However, there is an increasing body of evidence that implicates ceramide-mediated signalling in the mechanism of tissue IR injury. Thus, changes in ceramide metabolism have been associated with tissue damage in various models of IR or hypoxia/reoxygenation (5-9). Furthermore, it has been demonstrated that myocardial IR increases tissue cytokines such as TNF-α and Il-1β in the myocardium, that these cytokines may contribute to depressed contractility, myocardial structural abnormalities, and in particular, to myocyte apoptosis (10-12), and that ceramide mediates the actions of TNF-α and other cytokines in cell injury (2). It is therefore plausible that it is the over-production of cytokines that increases myocardial ceramide concentration via the modulation of activities of the enzyme(s) responsible for ceramide production or metabolism, thus leading to myocardial IR injury. Of note, IPC has been found to attenuate IR-induced over-production of TNF-α in the heart (24), and this study demonstrates that IPC prevents IR-induced over-production of ceramides as well. In addition, IPC has been shown to prevent IR-induced cardiomyocyte apoptosis (25-27), the process for which TNF-α and ceramide may serve as a principal mediators (7, 33, 34). Altogether, these results support the hypothesis that it is the increased accumulation of some specific ceramide(s) that serves a role of the intermediate signal between stress signals such as cytokines and various forms of IR-induced myocardial injury. We speculate that the prevention of this accumulation adds, at least partially, to the mechanism of myocardial protection afforded by IPC.

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