J Mol Cell Cardiol **34**, 509–518 (2002)
doi:10.1006/jmcc.2002.1533, available online at http://www.idealibrary.com on IDEAL®

Identification of a Novel Role for Sphingolipid Signaling in TNFα and Ischemic Preconditioning Mediated Cardioprotection

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(Received 27 December 2001, accepted for publication 24 January 2002)

S. Lecour, R. M. Smith, B. Woodward, L. H. Opie, L. Rochette and M. N. Sack. Identification of a Novel Role for Sphingolipid Signaling in TNFα and Ischemic Preconditioning Mediated Cardioprotection. *Journal of Molecular and Cellular Cardiology* (2002) **34**, 509–518. TNFα administration mimics ischemic preconditioning and neutralizing antibodies to TNFα and IL-1β abolish exercise-induced preconditioning. However, the pharmacology of TNFα's cardioprotective effects and associated downstream signaling events has not been delineated. We evaluated the temporal and dose specific requirements of TNFα to function as a preconditioning mimetic. Furthermore we postulated that the preconditioning effect of TNFα might be orchestrated via sphingolipid signaling. The cardioprotective effect of TNFα and the role of sphingolipid signaling were assessed using a classical preconditioning protocol in the isolated perfused rat heart with the measurement of infarct size and contractile function modulation in response to index ischemia and reperfusion. Recombinant TNFα at an optimal dose of 0.5 ng/ml mimicked ischemic preconditioning by reducing infarct size by 60% *v* non-preconditioned ischemia-reperfusion controls ($P<0.01$). The infarct sparing effect of TNFα required a wash-out period prior to the index ischemic-reperfusion. Moreover, the classic ischemic preconditioning antagonist such as 5-hydroxydecanoate abolished TNFα preconditioning. An inhibitor of the sphingolipid signaling pathway, N-oleoylethanolamine (NOE, 1 μM) attenuated ischemic and TNFα preconditioning. Likewise, cell-permeable C_2-ceramide and sphingosine 1-phosphate (sphingolipid signaling intermediates) both reproduced the preconditioning cardioprotective phenotype. Finally, TNFα and ceramide conferred preconditioning-like cardioprotection against post-ischemic contractile dysfunction and this cardioprotective effect was attenuated by NOE. In contrast, NOE did not reverse ischemic preconditioning enhanced post-ischemic contractile function. In conclusion, TNFα activates preconditioning-like tolerance against infarction and contractile dysfunction. This cardioprotection is mediated, in part, via activation of novel sphingolipid signaling intermediates.

Key Words: TNFα; Sphingolipid signaling; N-oleoylethanolamine; Infarct size; C_2-ceramide.

Introduction

Preconditioning with short episodes of non-lethal ischemia augments innate cardiac resistance towards subsequent ischemic injury.[1–3] Interestingly, the pleiotropic cytokine TNFα has been shown to evoke preconditioning in rats and rabbits.[4–7] Moreover, we have demonstrated that TNFα is necessary for ischemic preconditioning in the isolated perfused mouse heart and that this cytokine

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0022–2828/02/050509 + 10 $35.00/0

is sufficient to activate this acute cardioprotective phenotype in the intact mouse heart.[8] The pharmacology and the signaling events initiated by TNFα binding to its cognate type II cell membrane receptors have not been extensively explored with respect to preconditioning activated cell survival signaling. In this regard, the TNFα interaction with its cell surface receptors is known to interact with the sphingolipid pathway by activating the neutral sphingomyelinase, an enzyme that hydrolyses sphingomyelin to generate the intracellular second messenger ceramide.[9] Ceramide, in the presence of ceramidase and sphingosine kinase, will then form sphingosine and sphingosine 1-phosphate. This sphingolipid signaling pathway leads to multiple biological actions including regulatory roles in apoptosis, growth, cell survival and inflammation.[10–12]

TNFα has been shown to promote divergent cellular effects spanning from cell survival to cell death in the heart (reviewed Ref. 13). These conflicting biologic effects may be mediated, in part, via activation of the sphingolipid signaling intermediates.[9,14] Hence, we hypothesized that TNFα may direct its preconditioning-like effects via sphingolipid signaling. In this study, we have focussed on characterizing the dose and temporal requirements of TNFα in the preconditioning program. Furthermore we evaluated the role of the sphingolipid signaling intermediate—ceramide—in preconditioning and finally we characterized the interplay between TNFα signaling and classical ischemic preconditioning activated cell signaling.

Of note, studies by numerous investigators have demonstrated that the sham-procedure to open the chest and a cardiac ischemia-reperfusion *in vivo* result in the acute and sustained activation of numerous cytokines, including TNFα, IL-1β and IL-6.[15–17] Hence, in order to dissect out the relative contribution of TNFα we have used a reductionist approach, i.e. the isolated perfused rat heart, to study TNFα and sphingolipid intermediate signaling in classical preconditioning. In this study we found that TNFα mimics ischemic preconditioning in a dose and temporal dependent manner. Moreover, we demonstrate that TNFα protects against post-ischemic infarction and contractile dysfunction. We also demonstrate that considerable overlap exists between classical ischemia and TNFα activated preconditioning with regard to intracellular signaling events. Finally, we identify a novel sphingolipid signaling cascade that seems to participate both in ischemic preconditioning and TNFα mediated cardioprotection against infarction in the isolated perfused rat heart.

Materials and Methods

All experiments were conducted on adult male Long–Evans rats weighing 250–300 g in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, Washington DC, 1996). The University of Cape Town Medical School Research Ethics Committee approved all experiments. Of note, rats used in experiments described in Figures 2 to 4 were obtained from Elevage Depre, France while all the other rats were obtained from the animal facility at the University of Cape Town, South Africa.

Isolated rat heart model

Rats were anesthetized with 60 mg/kg intraperitoneal sodium pentobarbitone and given an intravenous injection of 200 IU heparin. Hearts were excised rapidly and placed in ice-cold Krebs–Henseleit perfusion buffer before being mounted on a Langendorff apparatus for perfusion at 37°C with Krebs–Henseleit buffer at constant pressure (100 cm H_2O). The buffer was equilibrated with 95% CO_2/5% O_2 and had the following composition (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.2 and glucose 11.0. For hearts subjected to a regional ischemia experiments, a 6/0 silk suture was placed around the left coronary artery to form a snare. A balloon was inserted through the left atrium into the left ventricle and the left-ventricular end diastolic pressure (LVEDP) was adjusted between 4 and 8 mmHg. Cardiac parameters were monitored continuously and included heart rate (HR), left ventricular developed pressure (LVDP: difference between left ventricular end systolic pressure and end diastolic pressure), the rate-pressure product [left ventricular developed pressure multiplied by the heart rate (RPP)] and the coronary flow (CF).

Experimental protocols

The perfusion protocol is shown in Figure 1. All hearts were allowed to equilibrate for at least 15 min and were consequently subjected to a standard 30 min of regional [Fig. 1(A)] or global ischemia [Fig. 1(B)] followed by 120 and 30 min of reperfusion respectively. IPC was elicited by two cycles of 5 min of global ischemia interspersed with 5 min reperfusion prior to the standard ischemia.

TNFα (from 0.05 to 5 ng/ml), the cell permeable c2-ceramide (1 μM) or sphingosine 1-phosphate

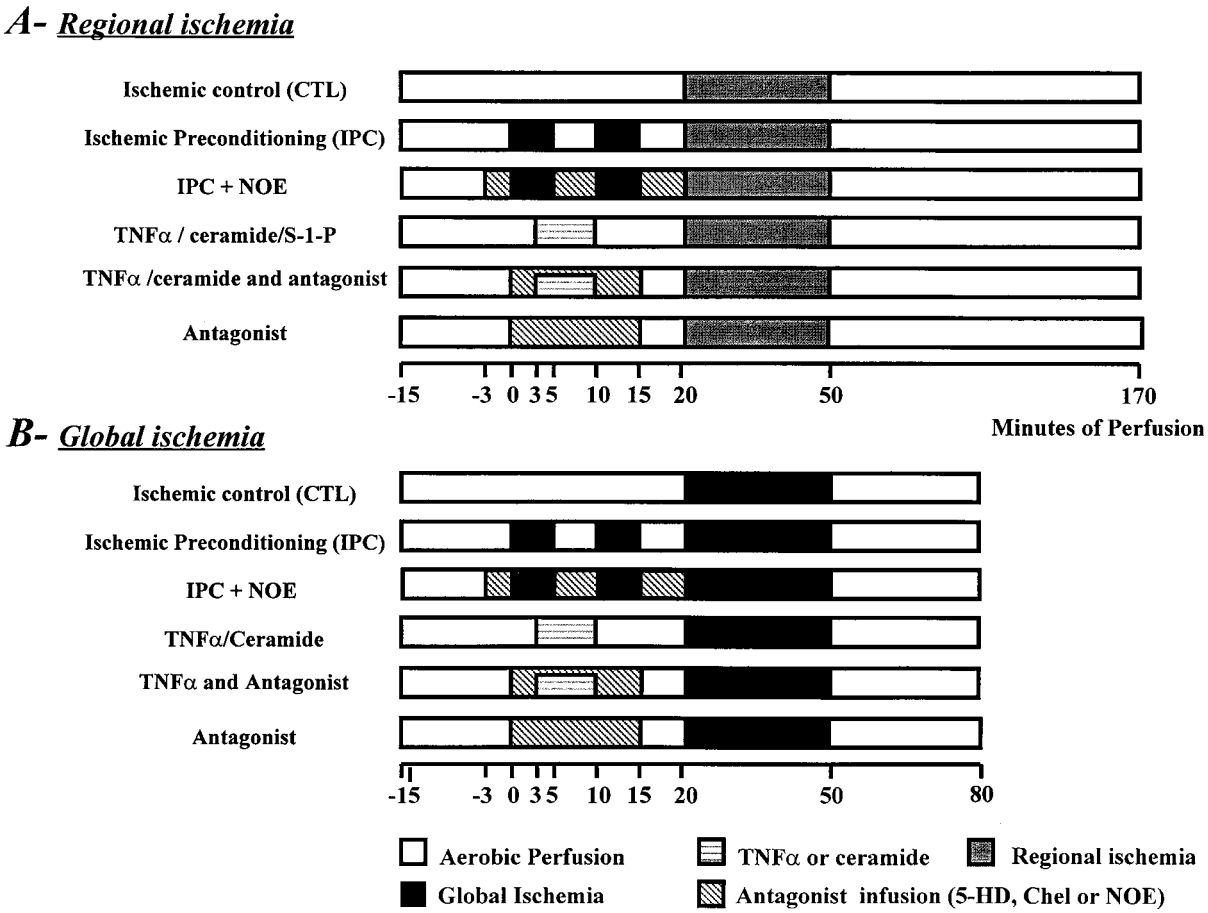

Figure 1 Experimental protocol conducted on an isolated rat heart model. All hearts underwent at least 15 min of a stabilization period. In part (A), hearts were subjected to 30 min of regional ischemia followed by 2 h reperfusion. In part (B), hearts were subjected to 30 min of global ischemia followed by 30 min of reperfusion. Hearts were preconditioned with two short episodes of global ischemia or pretreated with either TNF (0.05 to 5 ng/ml) or ceramide (1 μM) in the presence or absence of 5-HD (100 μM), chelerythrine (10 μM) or NOE (1 or 5 μM).

(10 nM) were given for 7 min followed by 10 min of reperfusion before the standard ischemia. Additional groups were perfused with 5-hydroxydecanoic acid sodium (5-HD; 100 μM), a blocker of the mitochondrial K_{ATP} channel[18] or N-oleoylethanolamine (NOE; 1 or 5 μM), a relatively specific inhibitor of the ceramidase[19] for 15 min followed by 5 min washout prior to the I/R insult. All drugs were infused directly above the heart alone or in the presence of TNFα (0.5 ng/ml), ceramide (1 μM) or an ischemic preconditioning stimulus. An additional group of hearts were pretreated with TNF (0.5 ng/ml) for 7 min and subjected directly to a standard ischemia–reperfusion period without any washout.

Measurement of the area of risk

In the regional ischemic model, the coronary artery was reoccluded at the end of the reperfusion period and a solution of 2.5% Evans blue was perfused to delineate the area of risk. Hearts were then frozen and cut into slices, incubated in sodium phosphate buffer containing 1% w/v triphenyltetrazolium chloride (TTC) for 15 min to visualize the unstained infarcted region. Infarct and risk zone areas were determined with planimetry and infarct was expressed as a percentage of the risk zone.

Pharmacologic agents

Recombinant rat tumour necrosis factor alpha was obtained from Pepro Tech EC Ltd (London, UK), 5-hydroxydecanoic acid was purchased from Research Biochemicals International (Natick, USA) and all other drugs were obtained from Sigma Chemical Company (St Louis, MO, USA).

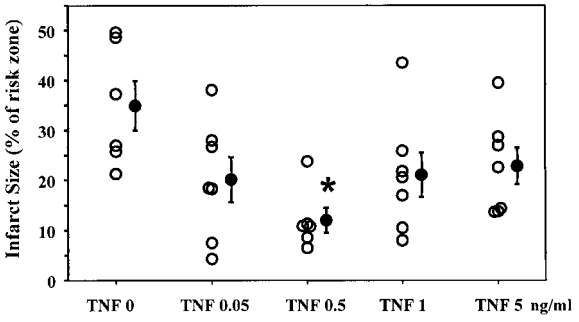

Figure 2 The dose-dependent cardioprotective effect of TNFα. Infarct size following a 30 min occlusion of the left anterior descending artery and 2 h of reperfusion. Infarct size was expressed as a percentage of the risk zone. Hearts were perfused with TNFα 0.05, 0.5, 1 or 5 ng/ml prior to ischemia. * $P<0.01$ *v* control group. $n=6$ in each group.

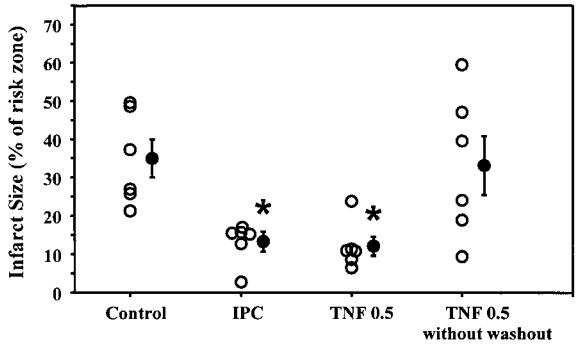

Figure 3 TNFα activated preconditioning. Infarct size following a 30 min occlusion of the left anterior descending artery and 2 h of reperfusion. Infarct size was expressed as a percentage of the risk zone. Hearts were preconditioned (IPC) with two short episodes of global ischemia or were perfused with TNFα 0.5 ng/ml with or without a washout of 10 min prior to the ischemia. * $P<0.01$ *v* control group. $n=6$ in each group.

Statistical analysis

All results are expressed as mean ± standard error of the mean ($n=6$ to 12 animals per group). For multiple comparisons, ANOVA followed by the Tukey's *post hoc* procedure was applied to isolate significant differences. A P value <0.05 was considered significant.

Results

Recombinant TNFα administration mimics ischemic preconditioning

To directly determine whether recombinant rodent TNFα administration could confer cardioprotection, various doses of TNFα were administrated to the perfused rat heart prior to the ischemia/reperfusion insult. In a range from 0.05 to 5 ng/ml TNFα tends to confer cardioprotection against infarct size (Fig. 2). The optimal TNFα dose of 0.5 ng/ml administered as a preconditioning-mimetic resulted in a significant reduction in infarct size compared with the control group (12.0 ± 2.5 *v* 34.9 ± 4.9, $P<0.01$). The protective effect afforded by TNFα was similar to the protective effect induced by ischemic preconditioning (12.0 ± 2.5 *v* 13.2 ± 2.7) (Fig. 3). When 0.5 ng/ml of recombinant TNFα was infused up to the moment of the index ischemic insult, i.e. no washout period, the infarct sparing effect was abolished, resulting in a similar size infarct to the ischemic controls (33.0 ± 7.7 *v* 34.9 ± 4.9, $P=$N.S., Fig. 3).

Ischemic preconditioning is known to be abolished by inhibition of mitochondrial K_{ATP} channel activation by co-administration of the mitochondrial K_{ATP} channel antagonist 5-hydroxydecanoate. Here, 5-HD completely blocked the cardioprotective effect of the preconditioning-mimetic effect of TNFα (infarct size TNFα − $12.0\pm2.5\%$ *v* TNFα + 5-HD − $28.5\pm3.9\%$, Fig. 4).

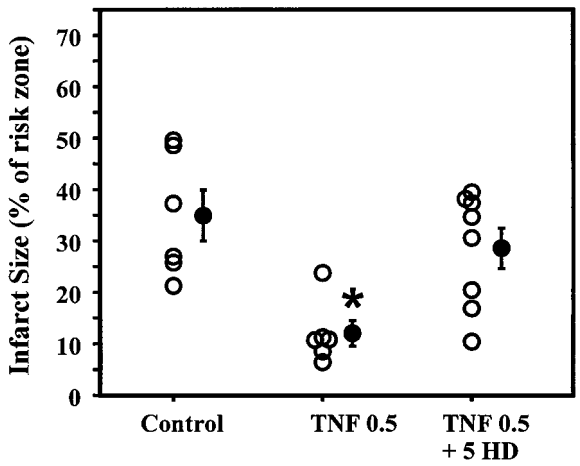

Figure 4 TNFα preconditioning requires activation of the mitochondrial K_{ATP} channel. Infarct size following a 30 min occlusion of the left anterior descending artery and 2 h of reperfusion. Infarct size was expressed as a percentage of the risk zone. Hearts were perfused with TNFα 0.5 ng/ml in the presence of 5-hydroxydecanoate acid (5-HD; 100 μM). * $P<0.01$ *v* control group. $n\geq6$ in each group.

Sphingolipid signaling in TNFα activated preconditioning

As a putative cytoprotective pathway downstream of TNFα induces sphingolipid second messenger

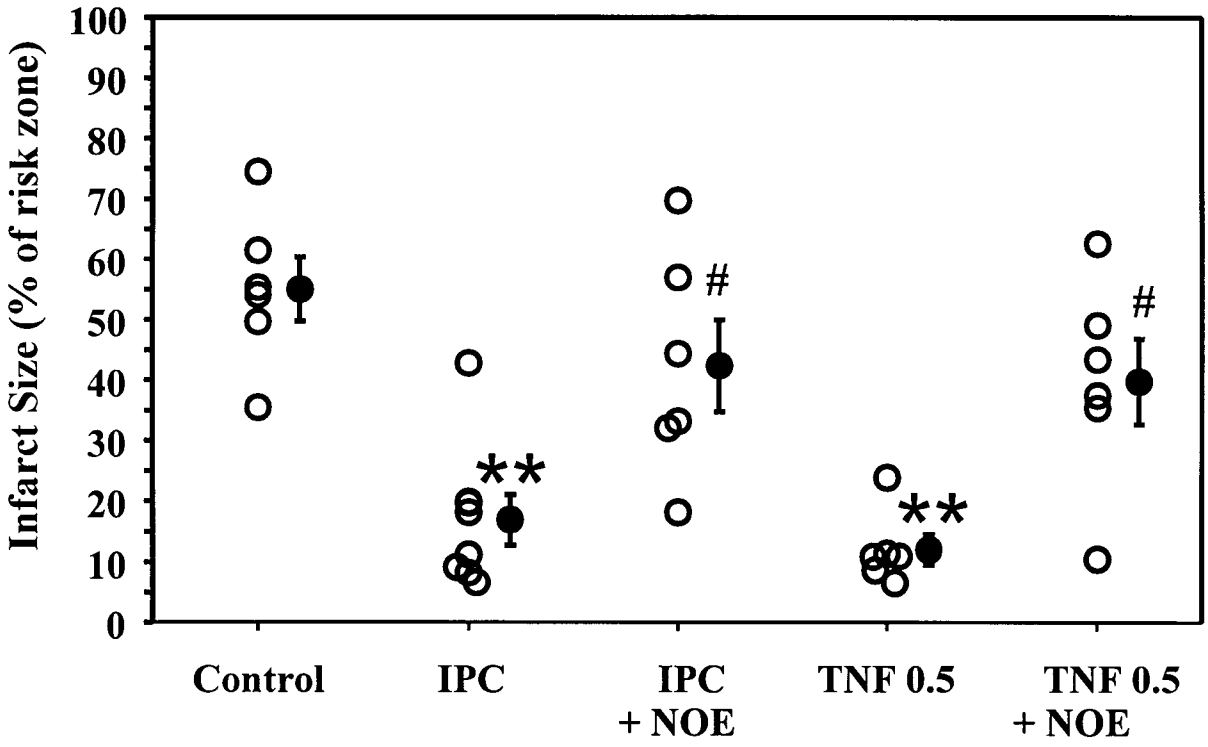

Figure 5 Role of the sphingomyelin pathway in ischemic and TNFα induced cardioprotection. Infarct size following a 30 min occlusion of the left anterior descending artery and 2 h of reperfusion. Effect of ceramide perfusion (1 μM) on the infarct size. Perfusion of NOE (1 or 5 μM) reduced the cardioprotection effect of IPC and TNFα. ** $P<0.01$ *v* control, # $P<0.05$ *v* respective preconditioning triggers. $n=6$ in each group.

mediated signaling[14] we began to interrogate this signaling pathway in our study. Interestingly, the ceramidase inhibitor, NOE (1 μM) abolished the infarct sparring effects of both ischemic preconditioning and of TNFα (Fig. 5). In addition, 1 μM of cell-permeable C_2-ceramide, a sphingolipid signaling intermediate significantly reduced the infarct size compared to non-preconditioned controls ($19.1\pm4.1\%$ *v* 55.0 ± 5.3, $P<0.001$, Fig. 6). This preconditioning-like effect of C_2-ceramide could be abolished by the ceramidase inhibitor, NOE. However, here a higher dose of NOE was required (5 μM), as compared to the dose required to inhibit the TNFα and ischemic preconditioning cardioprotective effects (1 μM) (Fig. 6). This discrepancy may be due of different pharmacokinetics between endogenous ceramide produced by TNFα and the exogenous C_2-ceramide. Of note, both doses of NOE have been used as ceramidase inhibitor in cardiovascular system.[19–21] As with the other preconditioning-like triggers, the proposed mitochondrial K_{ATP} channel antagonist, 5-hydroxydecanoate also abolished the preconditioning-like effect of ceramide (Fig. 6). Perfusion of sphingosine 1-phosphate (10 nM)[22] prior to the ischemia/reperfusion insult conferred cardioprotection against infarct size in a similar extent to ceramide ($P<0.001$) (Fig. 6).

TNFα activated preconditioning enhances post-ischemic ventricular functional recovery

As protection against stunning is an additional relevant end-point of preconditioning[23] we evaluated the role of the optimal dose of TNFα (0.5 ng/ml) on contractile recovery following global ischemia in the isolated perfused rat heart. The ischemic control group resulted in a marked decrease in the LVDP, RPP and the coronary flow compared to the pre-ischemic values (hemodynamic data shown in Table 1). The IP "trigger" resulted in augmented LVDP ($P<0.001$), in an improved RPP (Fig. 7) ($P<0.001$) and an increase in coronary flow ($P<0.001$) after 30 min of reperfusion compared to controls.

Addition of recombinant TNFα, 0.5 ng/ml did not modify the hemodynamic parameters before ischemia but resulted in a greater LVDP ($P<0.001$) and functional recovery (Fig. 7) ($57.6\pm3.2\%$ *v* $20.0\pm3.4\%$ for controls, $P<0.001$) after the index I/R compared to the ischemic controls.

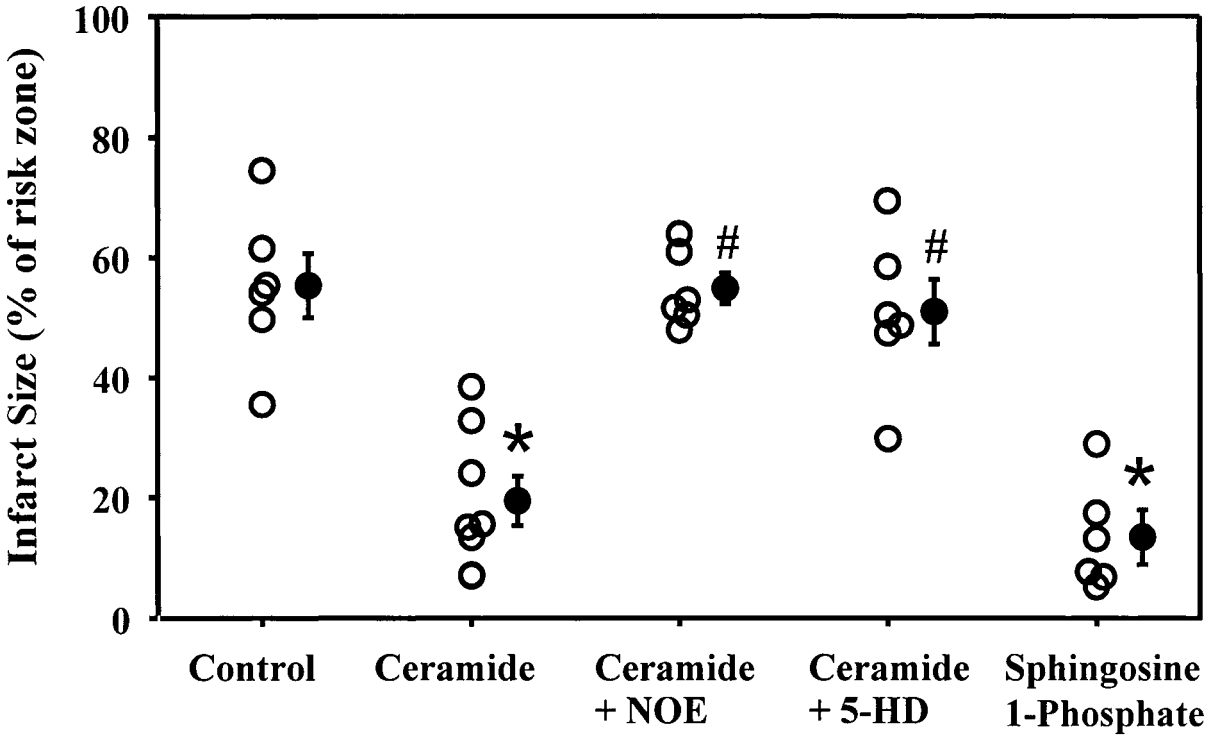

Figure 6 Ceramide and sphingosine 1-phosphate can induce a preconditioning-like cardioprotection. Infarct size following a 30 min occlusion of the left anterior descending artery and 2 h of reperfusion. Effect of ceramide (1 μM) or sphingosine 1-phosphate (10 nM) perfusion on the infarct size. Perfusion of NOE (5 μM) or 5-HD (100 μM) reduced the cardioprotective effect of ceramide. * $P<0.001$ *v* control, # $P<0.001$ *v* ceramide. $n \geq 6$ in each group.

Perfusion of 5-HD (100 μM) alone had no significant effect of hemodynamic functions in hearts subjected to an I/R insult. However, co-administration of 5-HD (100 μM) in the TNFα preconditioned hearts abolished the protective effects measured following the index I/R insult. The LVDP decreased from 62.9 ± 3.5 mmHg to 22.3 ± 2.7 mmHg ($P<0.001$) and the functional recovery decreased from $57.6 \pm 3.2\%$ to $13.2 \pm 1.9\%$ ($P<0.001$). We have previously demonstrated the ability of 5-HD administration to abolish the protection conferred by ischemic preconditioning in this model.[24]

Addition of the ceramidase inhibitor NOE (1 μM) 15 min before ischemia did not modify the coronary flow and the LVDP of the heart, during the perfusion or after 30 min of reperfusion. However, co-perfusion of NOE with 0.5 ng/ml TNFα significantly abolished the effect of TNFα and decreased the LVDP ($P<0.01$) and functional recovery (Fig. 7) ($P<0.001$) after 30 min of reperfusion compared to the hearts pretreated with TNFα alone.

Perfusion of C_2-ceramide (1 μM) did not modify the hemodynamics parameters of the heart before global ischemia insult but resulted in a greater LVDP during the reperfusion period compared to the ischemic control hearts ($P<0.001$). Co-perfusion of NOE (5 μM) with C_2-ceramide abolished the protective effect of C_2-ceramide (Table 1).

Discussion

In this study we demonstrate that TNFα mimics classical ischemic preconditioning and has both an infarct sparing effect and contributes towards improved post-ischemic functional recovery in the isolated perfused rat heart. Moreover, pharmacologic antagonist studies support a role for TNFα activated preconditioning through the classical preconditioning mediated mitochondrial K_{ATP} channel activation. Concurrently, the role of sphingolipid intermediate signaling is suggested by the abrogation of TNFα cardioprotective effects with N-oleoylethanolamine and due to the ability of cell-permeable C_2-ceramide and sphingosine 1-phosphate to parallel TNFα effects. Finally, as NOE attenuates ischemic preconditioning-directed infarct sparing effects but not its contractile recovery effects, putative alternate regulatory pathways may drive these divergent end-points of ischemic preconditioning.

Table 1 Pre- and post-ischemic hemodynamic parameters in the group of hearts exposed to global ischemia and reperfusion [protocol: Fig. 1(B)]

	Baseline	Pre-ischemic	Reperfusion 5 min	Reperfusion 10 min	Reperfusion 15 min	Reperfusion 20 min	Reperfusion 30 min
LVDP							
Control	109.0±6.0	119±6.9	16±3.0	17.0±2.7	21.7±2.9	25.0±3.0	28.0±4.4
IPC	114.3±6.5	121.7±5.8	43.4±8.9*	55.4±9.1**	66.6±9.1***	81.1±4.5***	86.3±3.8***
IPC+NOE	113.4±4.2	123.1±5.2	24.1±4.8	39.9±4.7*	55.6±8.2**	72.3±3.3***	78.0±4.4***
TNF 0.5	108.9±5.1	110.3±5.0	25.1±3.0	24.6±2.6	36.9±5.1	51.4±5.7*	62.9±3.5***
TNF 0.5+5-HD	119.7±4.7	124.7±2.6	5.7±4.9	7.7±6.3	12.7±5.5	14.7±6.5#	22.3±8.0###
TNF 0.5+NOE 1	105.3±3.5	108.7±5.7	14.7±1.3	15.3±2.4	18.3±3.4	22.0±3.1	31.7±4.1
Ceramide	101.0±3.9	96.0±4.2	73.0±7.3***	75.0±8.9***	76.0±9.7***	81.5±11.3***	93.0±9.3***
Ceramide+NOE 5	100.5±6.9	101.0±9.9	13.0±3.4	9.0±3.1	9.5±3.6	13.0±4.51	37.0±16.8
5-HD	114±4.5	116.3±4.3	14.0±3.3	15.3±4.5	19.0±5.5	18.9±7.3#	27.0±6.7##
NOE1	102.5±6.7	110±3.5	16.0±1.8	16.0±5.4	18.5±5.2	25.5±8.9	33.0±9.0
Heart rate							
Control	300±9	300±13	247±7	243±17	233±16	250±14	247±7
IPC	307±11	308±13	267±8	267±8	310±19	297±17	287±15
TNF 0.5	306±7	306±7	214±25	254±9	243±16	274±14	300±14
TNF 0.5+5-HD	320±21	313±12	180±31	217±34	220±35	227±25	223±24
TNF 0.5+NOE 1	327±12	307±13	184±25	200±20	227±38	217±21	243±11
Ceramide	295±17	300±20	210±13	245±5	250±6	250±10	260±14
Ceramide+NOE 5	295±10	285±10	230±44	240±41	260±32	235±32	245±22
5-HD	307±17	317±11	180±15	177±27	180±23	190±26	247±25
NOE 1	300±12	300±20	230±34	270±30	275±29	275±29	260±20
Coronary flow							
Control	12.6±0.6	12.6±0.7	7.1±0.6	7.0±0.7	6.5±0.7	6.5±0.7	6.5±0.9
IPC	12.7±1.0	15.7±0.2**##	10.4±1.0*#	12.1±0.7***#	11.9±1.1***#	12.3±1.1***#	11.7±0.9***#
TNF 0.5	12.2±0.6	12.1±0.5	8.6±0.6	9.6±0.6*	9.1±0.5*	9.1±0.5*	9.0±0.4*
TNF 0.5+5-HD	14.0±0.9	14.4±0.6	5.7±0.4#	5.9±0.5#	6.3±0.6#	6.1±0.5#	6.3±0.5#
TNF 0.5+NOE 1	12.3±0.9	11.2±0.7	9.1±1.1	8.1±0.4	8.1±0.5	7.6±0.5	7.3±0.6
Ceramide	14.5±1.3	14.5±1.3	15.5±0.5***	15.0±0.6***	14.5±0.5***	13.5±0.5***	13.5±0.5***
Ceramide+NOE 5	13.0±2.2	12.7±2.1	9.7±0.8	9.0±0.9	9.0±1.0	9.0±1.1	9.0±0.9
5-HD	13.2±0.8	13.5±0.9	4.8±1.0	5.7±1.1	5.7±1.2	5.8±1.2	5.9±1.2
NOE 1	11.9±0.4	11.2±0.7	9.1±1.1	8.1±0.4	8.1±0.5	7.6±0.5	7.3±0.6

Hemodynamic parameters measured after stabilization (baseline), prior to index ischemia (pre-ischemic) and at 5, 10, 15, 20 and 30 min of reperfusion. TNFα (0.5 ng/ml), ceramide (1 μM), 5-HD (100 μM), NOE (1 or 5 μM). LVDP = left ventricular developed pressure. # *v* TNF and * *v* controls. * 0.05, ** <0.01, *** <0.001, # <0.05.

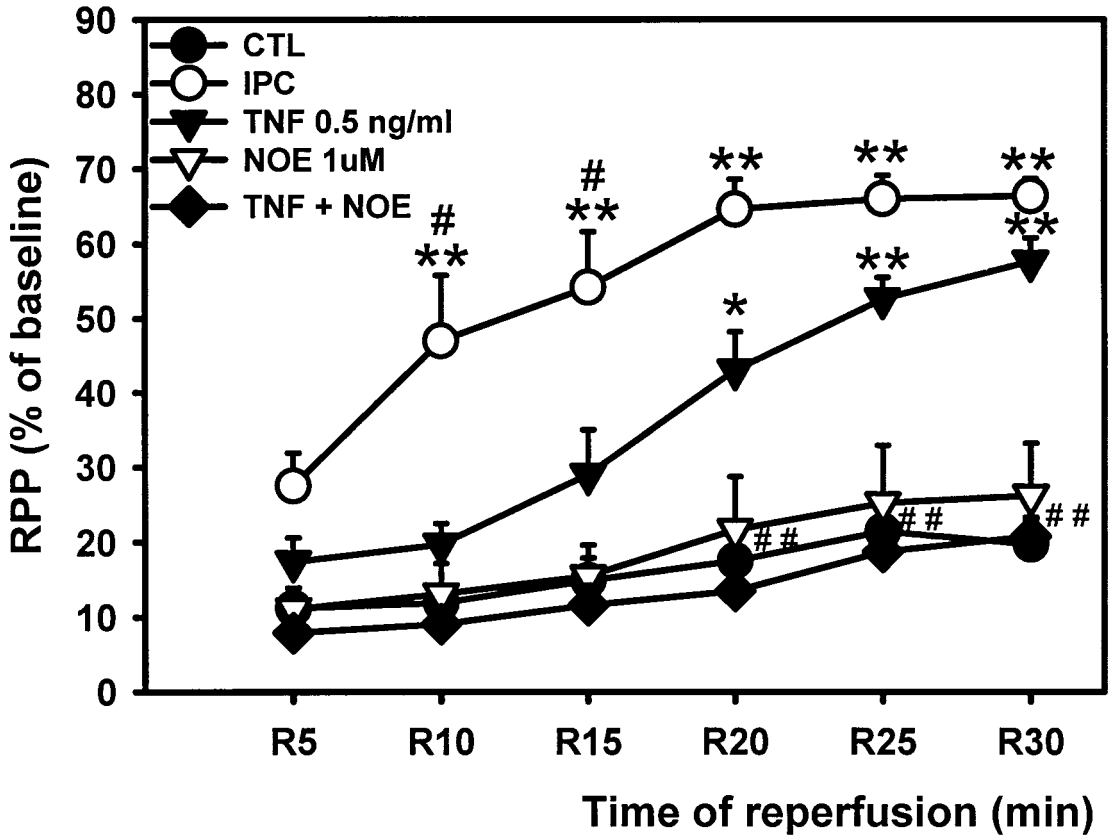

Figure 7 Rate pressure product (RPP) in response to modulation of TNFα activated signaling in preconditioning. Effect of 5-HD (100 μM) in the presence or absence of TNFα (0.5 ng/ml) on rate pressure product (RPP after 30 min reperfusion expressed as a percent of the value after the stabilization period) in isolated rat hearts subjected to an I/R insult (30 min of global ischemia followed by 30 min of reperfusion). * $P<0.01$, ** $P<0.001$ *v* control and # $P<0.01$, ## $P<0.01$ *v* TNFα.

A central role for reactive oxygen species in the induction of preconditioning is becoming increasingly apparent.[25–27] As TNFα is known to be upregulated by oxidative stress,[14,28,29] it should not be surprising that TNFα plays a role in the preconditioning program. Likewise, TNFα biosynthesis has been indirectly associated with ischemic preconditioning in the intact rabbit.[30] Using recombinant TNFα administration we also demonstrate that this cytokine mimics preconditioning in that a washout period is required prior to the index ischemia to activate the cardioprotective preconditioning phenotype. Hence, in parallel with the other adaptive effects of TNFα in response to biomechanical stresses in the heart,[31–33] TNFα signaling may be a representative of the innate-immune response to oxidative stress produced by ischemia preconditioning.

The pleiotropic effects of TNFα signaling result from ligand-induced trimerization of TNFα receptors with recruitment of a multitude of adaptor proteins, that in turn activate divergent signaling cascades (reviewed Refs 13, 34). As TNFα signaling can activate PKC and MAPK signaling,[35,36] the finding that the conventional pharmacologic antagonists of preconditioning such as 5-hydroxydecanoate abolish TNFα preconditioning is not unexpected. The fact that TNFα pretreatment without being washed out prior to ischemia/reperfusion did not confer cardioprotection may be explained via a toxic effect of TNFα during the ischemic period. Concurrently sphingolipid intermediates have been identified as TNFα activated signaling molecules that have recently emerged as putative modulators of cell survival programs.[14,37] Here, TNFα is a known agonist of sphingomyelinase, a family of enzymes that hydrolyse sphingomyelin to generate the lipid second messenger ceramide. Ceramide, in turn, in parallel to TNFα has strikingly different biologic effects that span from the promotion of cell survival to the promotion of apoptosis.[12,14,38] In this study we demonstrate that both C_2-ceramide and sphingosine 1-phosphate mirrored the cardioprotective effects of TNFα and that the ceramidase antagonist, NOE[20,21]

abolished the cytoprotective effects of TNFα and that of ischemic preconditioning. The dose of 1 μM C_2-ceramide used in this study was established as being optimally cytoprotective in our cell-culture system of preconditioning[39]—data not shown. Collectively, these data support the role for sphingolipid signaling in the promotion of cardioprotection in the preconditioning program.

A discrepancy in the role of sphingolipid signaling is evident concerning its requirement in ischemic preconditioning when we measure the effect of NOE on infarct size and on contractile recovery. The fact that NOE partially attenuated the infarct sparring effect of ischemic preconditioning, but not the contractile recovery effect suggest that alternative pathways evoke ischemic preconditioning protection against infarct size and contractile recovery. This concept is not unprecedented, in that Bolli and colleagues have noted a differential role of K_{ATP} channel activation on myocardial stunning and infarct size in rabbits.[23] This result also suggests that TNFα and ischemic protection against contractile recovery may act via distinct pathways from those required to promote cell survival alone. This hypothesis can be reinforced by the different profiles of the rate pressure product curves between TNFα and IPC groups.

The isolated perfused heart preparation was used in this study as this enabled us to dissect out the distinct temporal and dose requirement of TNFα in preconditioning independent of the known activation of the cytokine cascade following surgical stress in the *in vivo* preparation.[16] However, the perfused heart preparation model does have limitations in that post-ischemic reperfusion can only be maintained for a few hours. This *ex vivo* time frame does not enable the heart to completely evolve into the post-infarct remodeled heart. However, our reductionist approach does identify the putatively novel sphingolipid signaling pathway in preconditioning, but needs to be coupled to further *in vivo* work to more comprehensively evaluate this lipid intermediate second messenger system in preconditioning.

In conclusion, our study demonstrates that TNFα, at a hemodynamically neutral concentration, functions as a preconditioning mimetic and confers protection against infarct size and contractile recovery in the isolated perfused rat heart. Moreover, our results demonstrate that the cardioprotective effects of TNFα seem to signal through mitochondrial K_{ATP} channel activation as well as via a novel sphingolipid signaling pathway. Additional characterization of this latter signaling cascade may provide new insight into the cellular mechanisms driving the cardioprotective phenotype activated by ischemic preconditioning.

Acknowledgements

This study was supported by a Wellcome Trust Equipment Grant, UK (MNS), and in part by the South African Medical Research Council. Dr Sandrine Lecour was funded by the Fondation Pour la Recherche Medicale, France.

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