Effects of tumour necrosis factor-α on left ventricular function in the rat isolated perfused heart: possible mechanisms for a decline in cardiac function

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1   The cardiac depressant actions of TNF were investigated in the isolated perfused rat heart under constant flow (10 ml min^{-1}) and constant pressure (70 mmHg) conditions, using a recirculating (50 ml) mode of perfusion.

2   Under constant flow conditions TNF (20 ng ml^{-1}) caused an early (<25 min) decrease in left ventricular developed pressure (LVDP), which was maintained for 90 min (LVDP after 90 min: control vs TNF; 110 ± 4 vs 82 ± 10 mmHg, $P<0.01$).

3   The depression in cardiac function seen with TNF under constant flow conditions, was blocked by the ceramidase inhibitor N-oleoylethanolamine (NOE), 1 μM, (LVDP after 90 min: TNF vs TNF with NOE; 82 ± 10 vs 111 ± 5 mmHg, $P<0.05$).

4   In hearts perfused at constant pressure, TNF caused a decrease in coronary flow rate (change in flow 20 min after TNF: control vs TNF; -3.0 ± 0.9 vs -8.7 ± 1.2 ml min^{-1}, $P<0.01$). This was paralleled by a negative inotropic effect (change in LVDP 20 min after TNF: control vs TNF; -17 ± 7 vs -46 ± 6 mmHg, $P<0.01$). The decline in function was more rapid and more severe than that seen under conditions of constant flow.

5   These data indicate that cardiac function can be disrupted by TNF on two levels, firstly *via* a direct, ceramidase dependant negative inotropic effect, and secondly *via* an indirect coronary vasoconstriction.

Keywords: TNFα; cardiac contractility; N-oleoylethanolamine; sphingosine; sphingomyelinase; coronary circulation; Starling curve

Abbreviations: bpm, beats per minute; iNOS, inducible nitric oxide synthase; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; NOE, N-oleoylethanolamine; SMase, sphingomyelinase; TNF, tumour necrosis factor-α

Introduction

Tumour necrosis factor-α (TNF) is an essential mediator of the profound cardiovascular changes observed during the shocked-state associated with bacterial sepsis, and its experimental counterpart, endotoxic shock (Tracey *et al.*, 1986; Mathison *et al.*, 1988). Indeed, immunization of mice against TNF can protect against a lethal injection of endotoxin (Beutler *et al.*, 1985). The negative inotropic action observed during septic shock is mediated by circulating substances capable of depressing myocyte performance *in vitro* (Parrillo *et al.*, 1985). Monoclonal TNF antibodies can protect against both the hypotension and the decline in cardiac output observed upon live *Escherichia coli* administration to baboons (Tracey *et al.*, 1987), and TNF administration to dogs has been shown to have negative inotropic actions (Pagani *et al.*, 1992; Murray & Freeman, 1996). High circulating levels of TNF have also been associated with a variety of cardiac diseased states which are independent of bacterial infections (Levine *et al.*, 1990; Latini *et al.*, 1994; Vaddi *et al.*, 1994; Arbustini *et al.*, 1991; Smith & Allen, 1992) while overexpression of TNF in the murine heart leads to dilated cardiomyopathy associated with ventricular hypertrophy (Kubota *et al.*, 1997).

TNF-induced cardiac depression has been shown to have at least two phases. There appears to be an immediate depression of contractility as well as a later phase of contractile dysfunction. Cytokines, either alone or in combination, have the ability to cause upregulation of inducible nitric oxide

synthase (iNOS) in cardiac myocytes (Schulz *et al.*, 1992; Balligand *et al.*, 1994; Pinsky *et al.*, 1995), and nitric oxide from iNOS can subsequently adversely affect cardiac function. This altered cardiac function has been observed both as a direct decrease in cardiac performance (Schulz *et al.*, 1995), and as decreased responsiveness of cardiac myocytes to β-adrenergic stimulation (Balligand *et al.*, 1993). By definition iNOS is not constituatively expressed, and the protein synthesis required for its expression can take several hours. Another delayed action of TNF in the heart may involve the release of endothelins, whereby prior treatment of rats with lipopolysaccharide or TNF has been shown to cause the release of endothelin which results in a subsequent coronary constriction (Hohlfeld *et al.*, 1995; Klemm *et al.*, 1995).

In contrast to the delayed cardiac depressant effects of TNF described above, an immediate negative inotropic action has been observed within minutes of treatment, this has proven more difficult to characterize. The acute negative inotropic action of TNF has been shown to be sensitive to nitric oxide synthase inhibitors (Finkel *et al.*, 1992). Another report presented evidence to suggest that the immediate depression of function seen with TNF was *via* a nitric oxide-mediated decrease in myofilament calcium responsiveness (Goldhaber *et al.*, 1996). However, other authors have shown TNF-induced alterations in myocyte calcium handling and depressed myocyte calcium transients which are independent of nitric oxide (Yokoyama *et al.*, 1993). The same group have recently shown the potential involvement of sphingosine in TNF-

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induced changes in calcium homeostasis, and subsequent negative inotropic action (Oral *et al.*, 1997). Another recent report from the same group shows that the involvement of sphingosine in TNF-mediated cardiac depression is also evident *in vivo*, and is accompanied by a ventricular remodeling characterized as left ventricular dilation (Bozkurt *et al.*, 1998). In addition to this, short term TNF treatment has been shown to inhibit L-type calcium currents in cardiac myocytes, this was also accompanied by a decrease in calcium transients (Krown *et al.*, 1995). Using a Langendorff perfused heart preparation under constant flow conditions we have recently observed another early action of TNF in the heart. This is an immediate coronary vasoconstrictor action which could also contribute to the cardiac depressant action of TNF (Edmunds & Woodward, 1998).

The sphingomyelinase (SMase) pathway has been recognized as a major signalling pathway of TNF (Kim *et al.*, 1991; Schutze *et al.*, 1992; Dressler *et al.*, 1992; Yang *et al.*, 1993), and this pathway has recently been demonstrated in cardiac myocytes (Oral *et al.*, 1997). Sphingomyelinase can be activated by TNF to cause the breakdown of the membrane bound phospholipid, sphingomyelin to its metabolite ceramide, which can be subsequently converted to sphingosine by ceramidase. Both ceramide and sphingosine have potential second messenger functions (Kolesnick, 1991; Hannun, 1994; Merrill *et al.*, 1997). Sphingosine can depress cardiac function by decreasing calcium-induced calcium release from the sarcoplasmic reticulum (Webster *et al.*, 1994), as well as by depressing the L-type calcium current (McDonough *et al.*, 1994). N-oleoylethanolamine (NOE) is a relatively specific inhibitor of the ceramidase enzyme (Sugita *et al.*, 1975), and is a compound which we have made use of in our studies.

In a previous report we have described a coronary constrictor action of TNF in the isolated perfused rat heart (Edmunds & Woodward, 1998). This appears to be mediated through an interaction between sphingosine and thromboxane A_2. In the present paper we provide evidence to show that the immediate negative inotropic action of TNF, observed in the isolated perfused rat heart also involves the sphingosine pathway. We also show that in a model where coronary flow is allowed to vary with changes in coronary tone, the vasoconstriction observed in our previous study can act in synergy with the direct negative inotropic actions of TNF to cause a further decrease in cardiac function.

Methods

Throughout this study male Wistar rats, 280–310 g, have been used. Animals were anaesthetized by an interperitoneal injection of sodium pentabarbitone (200 mg kg^{-1}) and then killed by cervical dislocation. The heart of each animal was isolated and perfused, *via* the aorta, according to the Langendorff technique under both constant flow and constant pressure conditions, with prefiltered, oxygenated (0_2 95%, CO_2 5%) Krebs-Henseleit solution of the following composition (mM): NaCl 118, NaHCO$_3$ 25, KCl 4.7, KH$_2$PO$_4$ 1.2, MgSO$_4$.7H$_2$0 1.2, CaCl$_2$.6H$_2$0 1.2 and D-glucose 11.6, pH 7.4 and a temperature of 37°C. A fluid filled clingfilm balloon was placed in the left ventricle, and left ventricular developed pressure (LVDP) was used as a measure of cardiac contractility. Left ventricular end diastolic pressure (LVEDP) was set between 5 and 10 mmHg, with the use of a 1 ml syringe connected to a micrometer head, allowing fine adjustment of the intraventricular balloon volume. Hearts were allowed to beat spontaneously throughout the experimental procedure.

All hearts were perfused in a non-recirculating system for 30 min, before being switched to a recirculating system, with a total volume of 50 ml, for the remainder of the experiment, which lasted 90 min. The reason for recirculating the Krebs buffer was to reduce the amount of TNF used during each experiment, as well as to allow the build up of possible metabolites released by TNF. The recirculating perfusate was constantly gassed with 95% O_2, 5% CO_2. Starling curves were performed after 15, 45 and 75 min, whereby, the intraventricular balloon was emptied then slowly filled, in 0.03 ml aliquots every 30 s, to see if TNF altered the Frank-Starling response. Hearts were rejected from this study if LVDP failed to reach 100 mmHg.

For constant flow studies, hearts were perfused with a flow rate of 10 ml min^{-1}. Coronary perfusion pressure (CPP) was recorded by a pressure transducer, and used as an index of coronary tone. In another set of experiments hearts were initially perfused under constant flow conditions (10 ml min^{-1}) before being switched to a constant head of pressure of 70 mmHg. In this situation coronary flow was measured using a T 206 Transonic Systems Inc. flow probe attached to the aortic canula and represented the volume of Krebs buffer perfused into each heart with time.

Recombinant human TNF, 20 ng ml^{-1} (stock solution: 100 μg ml^{-1} in phosphate buffered saline with 0.1% bovine serum albumin) was added to the perfusate at 25 min, 5 min prior to recirculation. In preliminary experiments a range of concentrations, up to 100 ng ml^{-1}, were investigated, and 20 ng ml^{-1} found to be optimum for cardiac depression (results not shown), this was a similar concentration to that used in other *in vitro* studies showing TNF-induced cardiac depression (Schulz *et al.*, 1995). The enzyme sphingomyelinase, (SMase, from *Bacillus cereus*) was added exogenously in one set of experiments to cause the breakdown of the phospholipid sphingomyelin. When added to the perfuate at 25 min, i.e. 5 min before recirculation, it showed a concentration dependant (concentrations ranging from $0.001-0.01$ u l^{-1}) negative inotropic action (results not shown). In subsequent experiments a submaximal concentration of SMase, 0.003 u l^{-1}, was added 5 min prior to recirculation.

All antagonists were added directly to the Krebs buffer after 5 min, to allow 20 min for equilibration before TNF addition. The ceramidase inhibitor NOE (1 μM, dissolved in ethanol) was used to block sphingosine production from the SMase pathway (Oral *et al.*, 1997). The non-specific nitric oxide synthase inhibitor, nitro-L-arginine (100 μM, dissolved in saline) was used to block any nitric oxide release. The cylooxygenase inhibitor, indomethacin (10 μM, stock solution dissolved in equimolar Na$_2$CO$_3$) was added to block prostanoid accumulation from TNF-induced AA release. Bosentan (3 μM, dissolved in saline) a combined endothelin A and B receptor antagonist (Clozel *et al.*, 1994) was used to block the actions of any TNF-induced endothelin release. Control experiments were conducted with all of the above agents in the absence of TNF. Control experiments were also conducted with vehicles for each of the drugs used, none of these altered cardiac function (data not shown).

A series of experiments was conducted to observe the actions of endothelin-1, 3 pM-3 nM, in hearts perfused under a constant head of pressure, in order to mimic the coronary constriction previously seen by us under constant flow conditions with TNF (Edmunds & Woodward, 1998), with the objective of demonstrating the dependency of LVDP on coronary flow.

In some of these constant pressure experiments, and despite having the heart surrounded by a heated water jacket,

coronary flow decreased to such an extent that there was a fall in cardiac temperature, as measured by a temperature probe inserted into the right ventricle. For this reason some experiments were performed in order to investigate the effect of drop in temperature from $37-33°C$ on cardiac contractility.

Statistics

All results are expressed as mean ± standard error of the mean. Statistical analysis was carried out, where appropriate, using one-way ANOVA coupled to either Dunett's test or Tukey's multiple comparison test. For non-parametric data a Mann-Whitney U test was performed. Analysis was completed by the use of a computer programme (Minitab 10 for Windows).

Materials

Bosentan was also a gift from Dr A.G. Roach (Rhone-Poulenc Rorer, Dagenham, U.K.). rhTNFα was a kind gift from Bayer (Slough, U.K.). All of the other drugs used in this study were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Results

Actions of TNF in the isolated rat heart perfused under constant flow conditions

It can be seen from the typical experimental trace shown in Figure 1a that in control hearts left ventricular developed pressure (LVDP) decreased only slightly throughout the 90 min perfusion period. Recirculation after 30 min had no direct effect on LVDP (LVDP before recirculation vs after recirculation: 127 ± 3 mmHg vs 120 ± 5 mmHg, $P = NS$, $n = 13$). Addition of TNF, 20 ng ml^{-1}, into the perfusate resulted in a depression in cardiac function which was evident within a few minutes of TNF addition (Figures 1b, 2 and 6). This depression became significant 25 min after TNF addition, and was evident for the remainder of the protocol (LVDP after 90 min: control vs TNF; 110 ± 4 mmHg vs 82 ± 10 mmHg, $P < 0.05$, $n = 10$ for TNF). TNF had no chronotropic effect, (heart rate after 90 min: control vs TNF; 285 ± 11 beats per minute (b.p.m.) vs 288 ± 12 b.p.m. $P = NS$).

Actions of SMase in the isolated rat heart perfused under constant flow conditions

When added 5 min prior to recirculation SMase caused a negative inotropic action (Figure 3). This depression in function was seen immediately upon addition of SMase and became significant after 15 min. SMase also caused coronary constriction (increase in CPP 20 min after SMase: control, $n = 7$, vs SMase, $n = 8$; 13 ± 5 mmHg vs 75 ± 20 mmHg, $P < 0.01$). SMase did not alter heart rate (heart rate after 90 min: control vs SMase; 285 ± 11 b.p.m. vs 273 ± 25 b.p.m., $P = NS$).

Effects of antagonists and inhibitors on changes in contractility caused by TNF and SMase under constant flow conditions

N-oleoylethanolamine (NOE) Inclusion of the ceramidase inhibitor NOE, 1 μM, into the perfusate 20 min before addition of TNF completely blocked the negative inotropic actions of the cytokine (Figure 2), without altering basal

Figure 2 A graph to show changes in LVDP, in hearts, which occur with time, for control ($n = 13$) TNF treated, 20 ng ml^{-1} ($n = 10$) and TNF in the presence of NOE, 1 μM ($n = 9$). *$P < 0.05$, **$P < 0.01$, TNF vs control. #$P < 0.05$, TNF with NOE vs TNF alone.
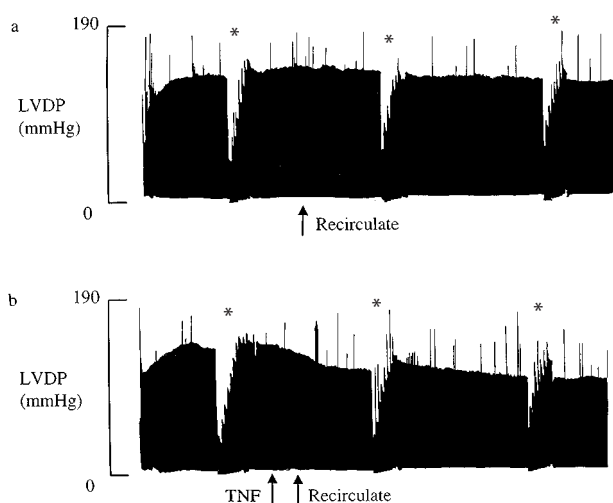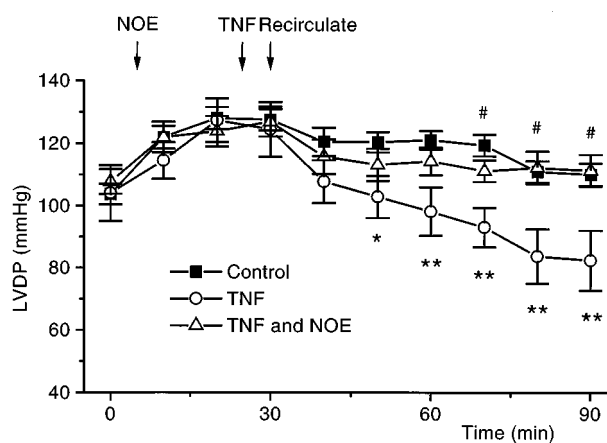
Figure 1 Experimental traces showing LVDP throughout the experiment. These traces are typical for (a) $n = 13$ control hearts and (b) $n = 10$ TNF, 20 ng ml^{-1} treated hearts. Starling curves were performed as indicated by *.
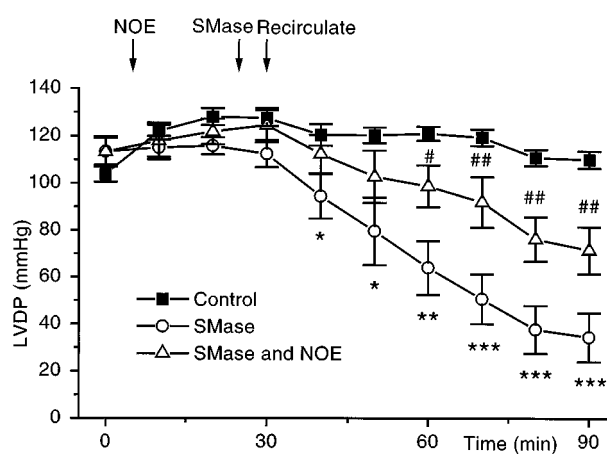
Figure 3 A graph to show the action of sphingomyelinase (SMase), alone and in combination with NOE, on the contractile parameters of the perfused heart. Control hearts ($n = 13$), SMase, 0.003 u l^{-1}, ($n = 7$). SMase, 0.003 u l^{-1} and NOE, 1 μM, ($n = 8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for SMase vs control. #$P < 0.05$, ##$P < 0.01$, for SMase with NOE vs SMase alone.

contractility (LVDP before NOE vs after NOE: 110 ± 4 mmHg vs 109 ± 4 mmHg, $P = NS$) or heart rate (heart rate before NOE vs after NOE: 267 ± 10 b.p.m. vs 263 ± 9 b.p.m., $P = NS$). NOE also caused a significant attenuation of the depression in function seen upon addition of SMase (Figure 3).

Nitro-L-arginine The nitric oxide synthase inhibitor, nitro-L-arginine, 100 μM, caused a rapid and sustained increase in coronary perfusion pressure (CPP before nitro-L-arginine vs 20 min after nitro-L-arginine: 56 ± 2 mmHg vs 126 ± 9 mmHg, $P < 0.001$). However, Figure 4 shows that nitric oxide synthase inhibition did not ameliorate the decreased contractility seen with TNF. Heart rate was not altered by nitro-L-arginine (heart rate before nitro-L-arginine vs after nitro-L-arginine: 289 ± 12 b.p.m. vs 290 ± 12 b.p.m., $P = NS$).

Indomethacin We have already reported that indomethacin was able to block the increase in CPP seen with TNF (Edmunds & Woodward, 1998). However, indomethacin did not alter the cardiac depression seen with TNF (LVDP after 90 min: indomethacin vs indomethacin and TNF; 101 ± 6 mmHg vs 66 ± 6 mmHg, $P < 0.01$). Indomethacin had no effect on basal parameters of LVDP (LVDP before indomethacin vs after indomethacin: 101 ± 4 mmHg vs 105 ± 4 mmHg, $P = NS$) or heart rate (heart rate before indomethacin vs after indomethacin: 263 ± 4 b.p.m. vs 278 ± 13 b.p.m., $P = NS$).

Bosentan Addition of the ET_A-ET_B-receptor antagonist bosentan (3 μM) to the perfusate did not block the TNF-induced alterations in cardiac function (LVDP after 90 min: bosentan vs bosentan and TNF; 103 ± 6 mmHg vs 73 ± 6 mmHg, $P = 0.01$). Bosentan also did not alter the basal LVDP (LVDP before bosentan vs after bosentan: 104 ± 2 mmHg vs 106 ± 2 mmHg, $P = NS$), heart rate (heart rate before bosentan vs after bosentan: 271 ± 9 b.p.m. vs 271 ± 6 b.p.m., $P = NS$) or CPP (CPP before bosentan vs after bosentan: 50 ± 2 mmHg vs 54 ± 3 mmHg, $P = NS$).

Actions of TNF in the isolated rat heart perfused under a constant head of pressure

We have reported earlier that TNF causes coronary constriction under constant flow conditions (Edmunds &

Woodward, 1998). Under constant pressure conditions this constriction would be expected to adversely affect cardiac contractility. A set of experiments were completed, where hearts were perfused under a constant head of pressure. Upon recirculation in control hearts there was a slight decrease in coronary flow, which continued throughout the experiment (Figure 5). Addition of TNF caused a more pronounced decrease in coronary flow (Figure 5). The LVDP in hearts perfused under a constant head of pressure follow the same pattern observed in hearts perfused under constant flow conditions. Namely there was a decline in LVDP after recirculation (Figure 6). However from Figure 6 it is clear that TNF caused a more pronounced decline in cardiac function under constant pressure conditions than with constant flow perfusion. Prior addition of NOE blocked both the decrease in coronary flow (Figure 5), as well as inhibiting the depression in cardiac function observed with TNF (Figure 7).
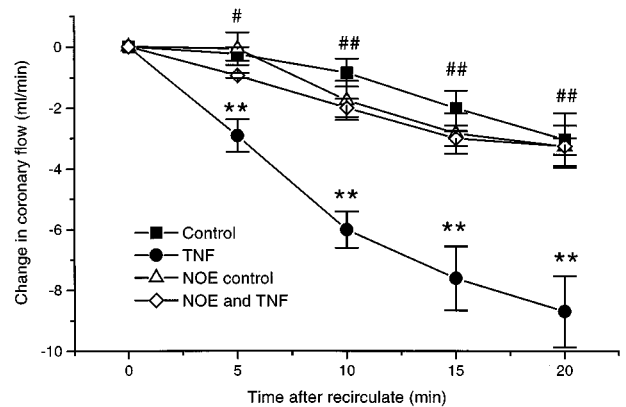
Figure 5 Effects of TNF (20 ng ml^{-1}) and NOE (1 μM), alone and in combination on coronary flow in hearts perfused at constant pressure ($n = 6-7$). **$P < 0.01$ TNF vs control, #$P < 0.05$, ##$P < 0.01$ NOE alone vs TNF with NOE. Initial coronary flow at 0 min: 12.4 ± 0.6 ml min^{-1}; 11 ± 1.2 ml min^{-1}; 8.9 ± 1 ml min^{-1} and 10.3 ± 2.4 ml min^{-1} for control hearts, TNF treated, NOE treated and NOE with TNF treated hearts respectively.

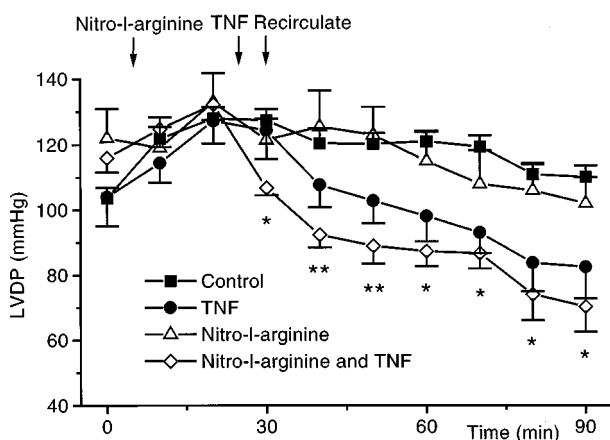**Figure 4** This graph shows the actions of nitro-L-arginine, 100 μM, on the depression in LVDP seen with TNF ($n = 8$). Control hearts ($n = 13$), TNF ($n = 10$), and control hearts in the presence of nitro-L-arginine ($n = 4$) are all shown. *$P < 0.05$, **$P < 0.01$, for TNF with nitro-L-arginine vs nitro-L-arginine alone.
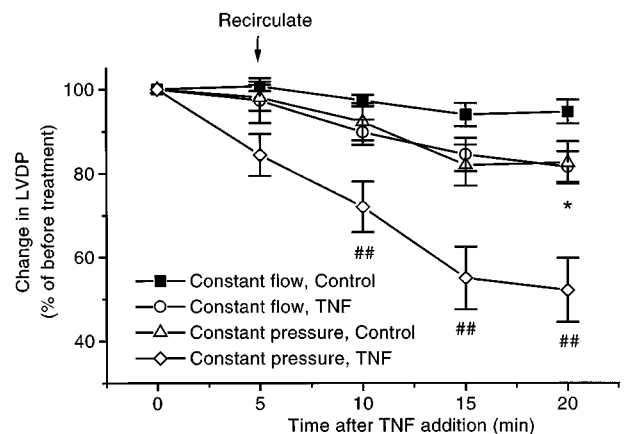
Figure 6 Effects of TNF, 20 ng ml^{-1}, in hearts perfused under constant pressure and constant flow conditions ($n = 6-7$). *$P < 0.05$, TNF vs control when perfused under constant flow conditions. *$P < 0.05$, TNF vs control when perfused under constant flow conditions. $P < 0.01$. TNF under constant flow conditions vs TNF under constant pressure conditions. Where initial LVDP at 0 min: 122 ± 4 mmHg; 126 ± 6 mmHg; 101 ± 6 mmHg and 106 ± 3 mmHg for constant flow control and TNF treated heart and constant pressure control and TNF treated hearts respectively.

Actions of endothelin-1 in hearts perfused under a constant head of pressure

Endothelin-1, when perfused through hearts under a constant head of pressure, showed a concentration dependant decrease in coronary flow, indicative of coronary constriction. This decrease in coronary flow was accompanied by a concentration dependant depression in cardiac function. At 1 nM, endothelin-1 caused a decrease in coronary flow from 9.5 ± 0.5 ml min^{-1} to 2.5 ± 0.7 ml min^{-1} ($P<0.001$, $n=5$). At the same concentration of endothelin-1 LVDP was depressed from 114 ± 9 mmHg to 56 ± 14 mmHg ($P<0.01$, $n=5$).

Effects of decreased temperature on cardiac function

A drop in temperature of the Krebs buffer from $37-33°C$ caused a decrease in heart rate, from 286 ± 9 b.p.m. to 201 ± 9 b.p.m. ($n=4$, $P<0.05$) and a concomitant increase in LVDP, from $123 \pm 2 - 133 \pm 5$ mmHg ($n=4$, $P<0.05$).

Starling curves

Figure 8 shows a Starling curve at 75 min (50 min after TNF addition), where LVDP increases with balloon volume. It can be seen that there is a tendency for TNF to decrease LVDP for a given balloon volume, although this did not prove to be a significant effect. However in hearts perfused under a constant head of pressure there was a significant depression in the hearts ability to perform Starling curves when in the presence of TNF (Figure 9). Under these conditions, prior addition of NOE was able to block these actions of TNF (Figure 9).

Discussion

The aim of this study was to examine the mechanisms underlying the early cardiac depressant actions of TNF in the isolated perfused rat heart. We have shown an early negative inotropic action which was exacerbated by the coronary constrictor actions of TNF. The fact that these effects of TNF can be attenuated by the ceramidase inhibitor NOE implicates sphingosine in these actions. The findings of the study presented herein can be summarized as follows: isolated hearts perfused under constant flow conditions with a pathophysiologically relevant concentration of TNF (Redl *et al.*, 1993), showed a depression in ventricular contractility, as indicated by the decrease in LVDP which was evident within minutes of TNF treatment and was sustained for the remainder of the experiment. There was also a tendency for TNF to cause a depression in the Starling curves under constant flow conditions, although this was not shown to be statistically significant. The direct depression in LVDP, under constant flow conditions, was not blocked by nitro-L-arginine, bosentan or indomethacin, which inhibit nitric oxide release, endothelin actions and arachidonic acid metabolism respectively (Gross *et al.*, 1990; Clozel *et al.*, 1994; Vane, 1971). Thus NO, endothelins and arachidonic acid metabolites are unlikely to be involved in the TNF-induced decrease in LVDP. The inability of indomethacin to block the direct depression in cardiac function is interesting as indomethacin did block the increase in coronary tone previously observed by us (Edmunds & Woodward, 1998). However, the negative inotropic actions of TNF were completely blocked by the ceramidase inhibitor
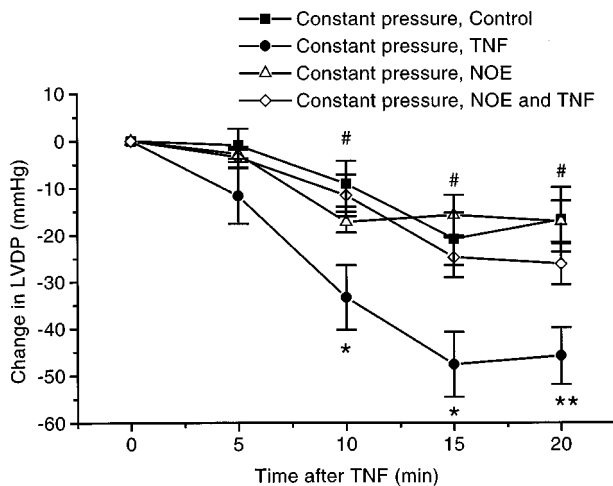

Figure 7 Effects of NOE on the depression in cardiac function seen with TNF, 20 ng ml^{-1} under constant pressure conditions. Shown as a change in LVDP after TNF administration ($n=6-7$). *$P<0.05$, **$P<0.01$ TNF vs control. #$P<0.05$ NOE vs TNF with NOE. Where LVDP at 0 min for NOE treated controls and NOE with TNF treated animals: 107 ± 6 mmHg and 100 ± 5 mmHg respectively.
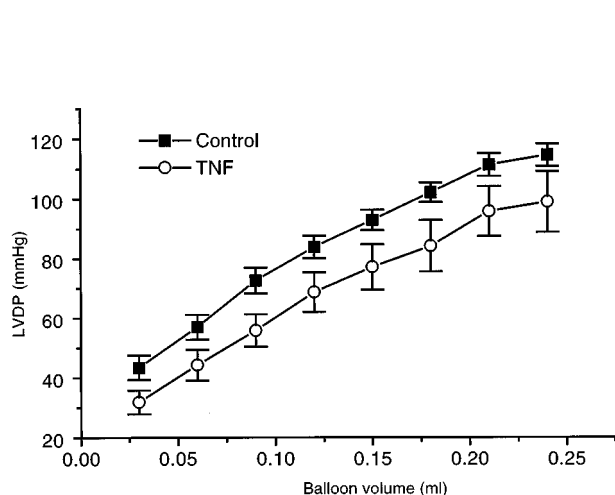

Figure 8 Starling curves for hearts perfused under constant flow conditions, 50 min after TNF addition. Control hearts ($n=13$), and TNF, 20 ng ml^{-1} ($n=10$).
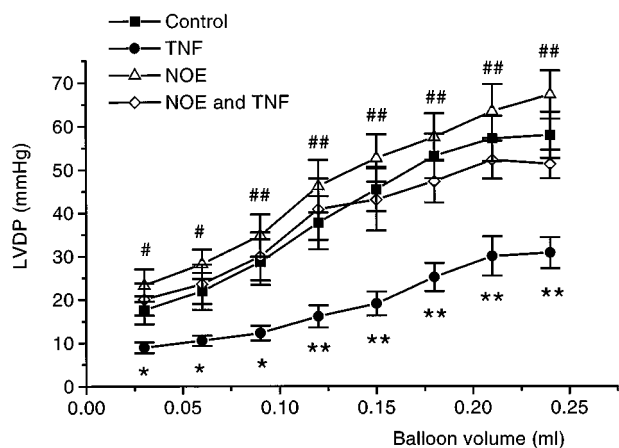

Figure 9 Starling curves in hearts perfused under a constant head of pressure, 50 min after TNF addition. Control hearts ($n=7$), TNF, 20 ng.ml^{-1}, alone ($n=7$), NOE, 1 μM, alone ($n=6$) and TNF with NOE. *$P<0.05$, **$P<0.01$, TNF vs control. #$P<0.05$, ##$P<0.01$, NOE alone vs TNF with NOE.

NOE. This suggests that these actions of TNF are, at least in part, dependant on ceramidase converting ceramide to sphingosine, and is in agreement with the recent data of Oral *et al.* (1997) and Bozkurt *et al.* (1998). The fact that NOE also attenuated the negative inotropic actions of SMase provides indirect evidence that activation of the sphingomyelinase pathway can depress cardiac contractility in the whole heart, and that this effect can be blocked with an inhibitor of the enzyme responsible for the conversion of ceramide to sphingosine.

We have previously reported that TNF causes coronary constriction in the rat heart (Edmunds & Woodward, 1998). The fact that TNF depressed cardiac contractility under constant flow conditions indicates that in this situation TNF is having a direct cardiac depressant action independant of changes in coronary flow. However, it is possible for the coronary circulation to alter cardiac function under constant flow condition *via* the 'garden hose' effect (Katz, 1992), which is something which we can not rule out. However, nitro-L-arginine also caused a vasoconstriction under constant flow conditions, but this was not accompanied by cardiac depression. This suggests that the garden hose effect is not important under these conditions and it provides evidence for a dissociation between cardiac depression and coronary constriction in this situation.

When hearts were perfused under a constant head of pressure, and coronary flow was allowed to vary, it was shown that TNF produced a greater, and earlier, depression of LVDP and of the Starling curves. This greater effect on contractility was coupled with a decrease in coronary flow to below $5 \, \text{ml min}^{-1}$. Therefore under these constant pressure conditions, the TNF-induced coronary constriction appears to be contributing to its enhanced depressant actions. The TNF-induced depression in LVDP could also be blocked by prior addition of NOE. These data extend our previous observations showing a role for the coronary constriction in the cardiac depressant effects of TNF. Both depressant actions, coronary (indirect) and cardiac (direct), appear to involve the sphingomyelin pathway, but the vasoconstriction was also dependant thromboxane A_2 (Edmunds & Woodward, 1998), however the direct action of TNF on cardiac function is independent of arachidonic acid metabolism as it was not inhibited by indomethacin. Therefore there appears to be a link between the sphingosine and cyclo-oxygenase pathways in the TNF-mediated coronary constriction, but the direct cardiac depressant actions of TNF are dependant on the conversion of ceramide of sphingosine, and do not involve the cyclo-oxygenase pathway.

The TNF-induced decrease in coronary flow observed under a constant head of pressure could be completely blocked by NOE. This constriction followed a very similar time course to that seen when hearts were perfused at a constant flow, the majority of the effect being within 10 min of TNF administration. It can be seen when comparing the graphs shown in Figures 6 and 7 that the earlier depression in LVDP seen in hearts perfused under a constant head of pressure paralleled this decrease in coronary flow.

The studies utilizing the vasoconstrictor peptide endothelin-1, also show that coronary constriction under constant pressure conditions has the ability to depress contractile function. This depression in cardiac contractility induced by endothelin-1 in the whole heart under constant pressure conditions is clearly outweighing the direct positive inotropic actions that endothelin-1 has on rat cardiac myocytes (Kramer *et al.*, 1991), adding further weight to the argument that the TNF-induced coronary constriction can contribute to depressed cardiac function.

Whilst activation of the sphingomyelinase pathway by TNF has been known for some time (Dressler *et al.*, 1992; Schutze *et al.*, 1992), this has only recently been shown in cardiac tissue (Oral *et al.*, 1997). These authors demonstrated that TNF-induced sphingosine accumulation was responsible for a negative inotropic action of TNF in isolated feline cardiac myocytes, our data supports this work and indicates that similar mechanisms exist in the rat heart.

The early depression in function was not dependent on nitric oxide because it was not altered by the nitric oxide synthase inhibitor nitro-L-arginine. This is in contrast to the observations of Finkel *et al.*, 1992, but is in agreement with other reports showing that the early direct decrease in cardiac function produced by TNF was not prevented by nitric oxide synthase inhibition (Yokoyama *et al.*, 1993; Oral *et al.*, 1997; Bozkurt *et al.*, 1998). Schulz *et al.* (1995) showed that TNF-induced and IL-1β-induced synthesis of iNOS was responsible for a late depression ($> 2 \, \text{h}$) in function in the isolated perfused rat heart. In contrast to our experiments, these authors did not observe an early depression in cardiac contractility, in fact a small increase in cardiac work was seen, we have no explanation for this apparent discrepancy. To our knowledge, alterations in cardiac function involving iNOS take place over a longer time course than used in our experiments (Schulz *et al.*, 1992; Szabo *et al.*, 1993; Shindo *et al.*, 1994; Balligand *et al.*, 1994; Pinsky *et al.*, 1995). Our results also show that the release of endothelin, which occurs after treatment of animals with lipopolysaccharide or TNF (Hohlfeld *et al.*, 1995; Klemm *et al.*, 1995), does not play a role in the early negative inotropic action of TNF. This is evidenced by the fact that the $ET_{A/B}$ receptor antagonist, bosentan, did not alter the TNF-induced decrease in LVDP.

The ceramidase inhibitor, NOE, which attenuated the depressant actions of TNF under constant flow and constant pressure conditions, disrupts signalling through sphingomyelin breakdown by inhibiting the conversion of ceramide to sphingosine (Sugita *et al.*, 1975), an action which appears to be specific for the SMase pathway (Coroneos *et al.*, 1995). This suggests that the depressant effect of TNF is due to increased sphingosine production. Sphingosine has been shown to have the ability to disrupt cardiac myocyte calcium handling, both by a decrease in calcium release from the sarcoplasmic reticulum (Sabbadini *et al.*, 1992, Dettbarn *et al.*, 1994; Webster *et al.*, 1994), and also by a depression in the L-type calcium current (McDonough *et al.*, 1994). We have shown that the addition of SMase also caused a decrease in cardiac function, however the site of action of this exogenous SMase was not known. The sphingomyelinase enzyme used has a molecular size of 25 kDa (Matsuyama *et al.*, 1992), so it is likely that it will have access to the cardiac myocytes from the coronary circulation. A potential limitation of this study is that the site of action of this enzyme was not defined and it may have been on the endothelial cells or the smooth muscle cells of the coronary circulation and not directly on the cardiac myocytes. However, the cardiac depression seen with SMase was still attenuated by NOE, suggesting activation of the SMase pathway, and release of sphingosine is responsible for its cardiac depressant action. However, it should be noted that the source of the SMase used in this study was bacterial in origin, and hence it is possible that it contained trace amounts of endotoxin. Contamination with endotoxin, and hence subsequent activation of constitutive nitric oxide synthase by endotoxin, could possibly explain why NOE only partially blocked the SMase induced depression in cardiac function.

When perfusing under a constant head of pressure, the coronary constriction caused the flow rate to decrease, and

despite the fact that the heart was surrounded by a water jacket at 37°C, a fall in temperature of the heart occurred. However in control experiment where hearts were perfused at 33°C, a temperature comparable to that seen when coronary flow was maximally decreased by TNF, heart rate decreased and LVDP actually increased. Therefore if this was to occur upon perfusion with TNF one would expect slight protection against the decrease in myocardial contractility, and this may have masked an even more severe depressant action of TNF.

In summary, we add further evidence in confirmation of the study by Oral *et al.* (1997), showing that a direct and early negative inotropic action of TNF in the isolated perfused rat heart is likely to be mediated *via* activation of SMase. Further to this, we have shown that the direct negative inotropic action of TNF can be accentuated by its coronary vasoconstrictor action.

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