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Endogenous nitric oxide induced by interleukin-1β in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay

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We have used the comet assay (single cell gel electrophoresis) to measure nitric oxide-induced DNA damage in rat islets of Langerhans and insulin-containing HIT-T15 cells. Damage was induced following treatment with the nitric oxide donor SIN-1, which also releases superoxide, but was not reduced by exogenous superoxide dismutase, suggesting that nitric oxide itself, rather than superoxide or peroxynitrite may be the active species. The DNA damaging effect of nitric oxide was easily detectable at the earliest time point tested (15 min). Damage also resulted following induction of nitric oxide synthase by the cytokine interleukin-1β in both islets and HIT-T15 cells and was prevented by replacing the substrate, arginine, with nitromonomethyl arginine. Thus intracellular levels of nitric oxide generated by interleukin-1β-induced nitric oxide synthase were sufficient to cause DNA damage in islet cells and HIT-T15 cells.

Interleukin-1β; Nitric oxide; DNA damage; Islets of Langerhans; HIT-T15 cell; Comet assay (single cell gel electrophoresis); Peroxynitrite

1. INTRODUCTION

Although exogenous nitric oxide generated under physiological conditions has been shown to cause DNA damage in mammalian cells [1], and macrophage-derived nitric oxide has been shown to produce DNA damage in single cells derived from rat islets of Langerhans [2], it is not clear whether induction of endogenous nitric oxide synthase in islet cells would be sufficient to produce detectable DNA damage. Nitric oxide is the product of the oxidation of arginine to citrulline, a reaction catalysed by the enzyme nitric oxide synthase [3]. Nitric oxide is a small lipophilic molecule, with a half-life of 5–30 s, which is likely to produce cytotoxic, mutagenic or second messenger effects in the cell in which it is produced, or in adjacent cells. It reacts rapidly with oxygen and oxygen radicals [4] to give rise to other species, such as peroxynitrite ($ONOO^-$) [5], (with a half-life 1–2 s) which are also thought to exert damaging effects [6]. Conditions favouring formation of peroxynitrite have recently been associated with neurotoxicity, whereas a change in the ambient redox state can result in a paradoxical neuroprotective effect of nitric oxide [7].

In this paper we have investigated DNA damage caused by nitric oxide, derived exogenously from the nitric oxide donor 3-morpholinosydnonimine (SIN-1), or endogenously by interleukin-1β-induction of nitric oxide synthase in insulin-containing cells. We also assess the role of superoxide and peroxynitrite in this system. Southern and co-workers first demonstrated that L-arginine-derived nitric oxide was responsible for the synergistic inhibition of insulin secretion by the combination of IL-1β and tumour necrosis factor-α (TNFα) and partly mediated the inhibitory effects of IL-1β alone [8]. Nitric oxide's role in the inhibition of insulin secretion by IL-1β has been confirmed [9,10]. There is evidence supporting IL-1β-increased expression of mRNA for inducible nitric oxide synthase in HIT-T15 cells [11], stimulation of radiolabelled arginine to citrulline conversion in RINm5F cells [12] and IL-1β-mediated nitrite formation in RINm5F cells [13] as well as cyclic GMP elevation in islets [14,15].

Free oxygen radicals have been suggested to mediate some of the toxic action of IL-1β or combined cytokines on pancreatic β-cells [16,17], and the generation of oxygen radicals by the enzyme xanthine oxidase caused islet cell lysis [18]. However, nitric oxide derived from activated macrophages has also been shown to promote islet cell lysis [19]. Kolb and co-workers have suggested that this macrophage-derived nitric oxide may be responsible for islet damage in Type 1 diabetes [20]. In a recent scientific letter, however, Mandrup-Poulsen et al. [21] have summarised evidence for a role of intracellular nitric oxide generated by inducible nitric oxide synthase in inhibiting β-cell function, and possibly facilitating destruction of β-cells. Interleukin-1β results in inhibition of islet cell function, but the extent to which it

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implements cell death/lysis and which free radicals are necessarily involved are unresolved issues.

We have used a modified version of the comet assay or single cell gel electrophoresis assay (SCG) [22,23] as a sensitive method for detecting DNA strand breaks, both those arising directly and those arising during the repair of damaged bases, together with alkali-labile lesions. This procedure resembles the in situ nick translation assay [2] insofar that it measures DNA strand breakage in individual cells. Two experimental approaches were used, the first was to discover if short-term incubation of islet cells or HIT-T15 cells in the presence of the nitric oxide donor 3-morpholinosydnonimine (SIN-1), produced DNA damage and whether superoxide dismutase protected cells against damage. Secondly, we wanted to see if IL-1β treatment resulted in measurable DNA damage and if nitromonomethyl arginine (NMMA) blocked such an effect. We measured the accumulation of nitrite (an oxidative product of nitric oxide) in the tissue culture medium from islets and HIT-T15 cells to confirm nitric oxide formation.

2. EXPERIMENTAL

2.1. *Materials*

Collagenase type XI, agarose and superoxide dismutase (SOD) were from Sigma Chemicals (UK), N^G monomethyl-L-arginine (NMMA) was from Calbiochem (UK). 3-Morpholinosydnonimine (SIN-1) (Cassella AG) and *S*-nitrosoglutathione (SNOG) were from Wellcome Research Labs, Beckenham (UK). Tissue culture medium (RPMI-1640), foetal calf serum, penicillin and streptomycin, Hank's balanced salt solution and trypsin were from Gibco (UK). Human recombinant interleukin-1β (code 86/552) was from National Institute of Biological Standards and Control (UK).

2.2. *Islet isolation and cell preparation*

Islets of Langerhans were isolated from Sprague–Dawley rats (200 g) by a modification of a collagenase digestion technique [24]. Islets were separated from pancreatic acinar tissue and were picked, using a finely drawn out Pasteur pipette, into a bicarbonate-buffered medium [25], pH 7.4, containing 2 mM glucose. Groups of 100 islets were cultured for 24 h in RPMI-1640 tissue culture medium supplemented with 11 mM glucose, 5% foetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) prior to all experiments. The treatments are described in the legends. For SIN-1 and superoxide dismutase treatments see Table I and for cytokine treatments see Fig. 1.

The hamster transformed β-cells, HIT-T15 (passage number 73-77) were trypsinised and plated into 2 cm^2 culture wells (4×10^5 cells) and cultured in 1 ml RPMI-1640 medium containing 5.5 mM glucose, foetal calf serum (10% v/v), penicillin (50 units/ml) and streptomycin (50 μg/ml) for 2 days prior to treatments (given in the legends).

2.3. *Measurement of DNA damage*

Groups of 100 islets were trypsinised into single cells and HIT-T15 cells were trypsinised off the culture wells. The comet assay was performed as described by Singh et al. [22] and modified by Green et al. [23]. Following the appropriate treatment (see Table I and Fig. 1), groups of single cells (5×10^4 per slide) were embedded on frosted slides, in duplicate, in a 0.6% agar layer (made up with RPMI + 10% foetal calf serum), on top of a 0.6% agar base layer, a third top layer consisting of 0.6% low melting point agar, was added. The cells embedded in agar were placed in an incubator at 37°C for 1 h, this was sufficient to allow repair of trypsin-induced damage. Slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Na-sarcosinate, 10% DMSO and 1% Triton) for 1 h to lyse away non-nuclear cell components and then placed in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 40 min, this allowed the DNA-containing strand breaks to unwind, and enabled the DNA fragments to move towards the anode, in a comet tail, during electrophoresis at 20 V for 24 min. The slides were neutralised and nuclei stained with ethidium bromide (20 μg/ml). DNA damage was indicated by the amount of DNA streaming from the nucleus. Quantitation was carried out by a fluorescent image analysis system which compared the overall length of intact nuclei versus damaged nuclei with a comet tail. At least 3 microscope field images or frames were captured per slide; therefore, in one experiment at least 6 frames were analysed per treatment and each frame contained on average 5–15 nuclei. The slide images were enhanced using an Optimax V image analysis system (Synoptics, Cambridge, UK) and the mean comet length for each treatment was determined [23].

2.4. *Determination of nitrite and nitrate*

Tissue culture medium from experiments involving treatment of intact cultured islets and HIT-T15 cells was assayed for nitrite and nitrate. To ascertain the proportion of nitrate relative to nitrite produced by cells, nitrate in the tissue incubation medium was reduced using cadmium powder and ammonium chloride/borax buffer [26] prior to analysis for nitrite. For the assay of nitrite 75 μl samples were mixed with an equal volume of Griess reagent (0.1% naphthyl ethylenediamine and 1% sulphanilamide in orthophosphoric acid, 1:1 v/v) [27] and absorbance read against standard sodium nitrite solutions (10–100 μM) at 546 nm. Where nitrite and nitrate were jointly determined, cells were incubated in physiological buffer [25], rather than RPMI-1640.

2.5. *Statistical analysis*

Values for nitrite, nitrate and comet length are means ± S.E.M. from at least three separate experiments unless otherwise stated. Statistical differences between mean values were determined by an unpaired Student's *t*-test, with the levels of significance established at $^*P < 0.05$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

3. RESULTS

3.1. *Nitric oxide and DNA damage*

Very significant DNA damage was observed after treatment of both islet and HIT-T15 cells with 100 μM SIN-1 for 30 min (Table I). We were able to detect DNA damage within 15 min exposure of HIT-T15 cells to SIN-1 at 100 μM, with an increase in mean comet length from 49.1 ± 1.8 to 62.3 ± 2.0, $^{***}P < 0.001$, $n = 6$, one experiment. Concentrations of SIN-1 up to 1 mM appeared to produce dose-dependent DNA damage but these were above levels that give a linear increase of apparent comet length with dose. Superoxide dismutase was used to test whether DNA damage might be arising from nitric oxide itself, or from peroxynitrite or other oxygen radicals formed from the superoxide released by SIN-1. In HIT-T15 cells superoxide dismutase (SOD) when used at 200 U/ml (Table I) or 20 U/ml (data not shown) did not affect comet formation. In combination with SIN-1 at 100 μM, superoxide dismutase 200 U/ml did not prevent DNA damage, indeed it appeared to enhance the effect of SIN-1 ($^+P < 0.05$). SIN-1 generated significant amounts of nitrite in HIT-T15 cell incubation medium in 30 min (3.4 ± 0.5 to 13.2 ± 0.8 μM), although a larger proportion of nitric oxide appeared

to be converted to nitrate, from 5.9 ± 1.5 to 54.8 ± 2.1 μM, $n = 6$, one experiment. Nitrite concentration was also increased in medium from cells incubated with SIN-1 in the presence of superoxide dismutase (21.7 ± 2.1 μM), but less nitrate was generated (25.4 ± 2.2 μM). The nitric oxide donor *S*-nitrosoglutathione (SNOG) (300 μM) is thought not to generate superoxide. Following a 30 min exposure of islets to SNOG, DNA damage was observed, with an increase in mean comet length from 50.5 ± 2.2 to 77.8 ± 6.8, $n = 6$ and a corresponding increase in nitrite from 1.1 ± 0.5 to 95.7 ± 2.9, $n = 3$, one experiment. Total islet and HIT cell protein content were not affected by treatments shown in Table I and Fig. 1 (results not shown).

3.2. *IL-1β and DNA damage*

Treatment of cultured islet cells and HIT cells with IL-1β (0.1 nM) for 18 h in the presence of arginine, resulted in significant DNA damage, in islets (Fig. 1A) and in HIT-T15 cells (Fig. 1B) in 4 separate experiments for each, as well as an increase in nitrite production by both cell types (Fig. 1C and D). In L-arginine-free medium supplemented with NMMA (1 mM), the effects of IL-1β on DNA damage (Fig. 1A,B) or nitrite production (Fig. 1C,D) were blocked vs. control for both islet and HIT cells. Although the overall mean comet length value appeared to be quite similar for both islet and HIT-T15 cells, examination of the percentage distribution of cells with a particular comet length (data not shown) reflected the fact that in isolated cultured islets the mean comet length was 40 μm for an intact islet cell nucleus but was 48 μm for the islet controls indicating there were always some control islet single cells that had produced comets. This may have been due to the heterogeneous composition of the islets or damage during the preparation of single cells. HIT-T15 cells had a larger average nuclear diameter (49 μm) compared to islet cells and the control cells were rarely cometed.

4. DISCUSSION

We have presented evidence suggesting that nitric oxide causes DNA damage in nondividing cells (islets) and in transformed dividing cells (HIT-T15 cells) and that L-arginine-derived nitric oxide is involved in mediating the effects of IL-1β on DNA damage in these cell types. It appears that nitric oxide itself, rather than peroxynitrite, may be the active DNA-damaging species.

DNA damage may be assessed by the direct detection of DNA single-strand breaks and alkali-labile sites by a variety of techniques (for review see [28]). The present study, using the comet assay, confirms the observation, using in situ nick translation, that islet DNA is a target for cytotoxic levels of nitric oxide [2]. However, the reports differ not only in the time points studied (here 15–30 min vs. 3 h) and the nitric oxide donor compound used (SIN-1 vs. sodium nitroprusside) but also in the nature of the DNA damage assayed. In the nick translation assay the DNA strand-breaking agent hydrogen peroxide (90 mM) is used as a standard treatment to remove endogenous peroxidase activity [29]. Presumably it is undetected by nick translation because the assay

Table I

DNA damage in islet and HIT-T15 cells following 30 min exposure to the nitric oxide donor SIN-1 and in the presence or absence of superoxide dismutase

Treatment	Overall mean comet length ± S.E.M. (*n*)		Nitrite (μM) ± S.E.M. (*n*)		Nitrate (μM) ± S.E.M. (*n*)	
(A) Islets						
Control	51.8 ± 1.8	(20)	2.4 ± 1.7	(9)	–	
SIN-1 100 μM	76.2 ± 3.2****	(17)	44.0 ± 3.2****	(9)	–	
(B) HIT-T15 cells						
Control	46.8 ± 1.4	(30)	1.2 ± 0.2	(15)	–	
SIN-1 100 μM	65.9 ± 2.4****	(29)	48.8 ± 2.9****	(15)	–	
(C) HIT-T15 cells						
Control	51.9 ± 2.2	(12)	3.4 ± 0.5	(6)	5.9 ± 1.5	(6)
SIN-1 100 μM	77.8 ± 3.6****	(12)	13.2 ± 0.8****	(6)	54.9 ± 2.1****	(6)
SOD 200 U/ml	50.3 ± 3.4	(12)	3.9 ± 0.4	(6)	2.5 ± 1.0	(6)
SOD + SIN-1	95.2 ± 5.5****,+	(12)	21.7 ± 2.1***,+	(6)	25.4 ± 2.2****,++++	(6)

Single cells obtained after trypsinising intact, freshly isolated islets or HIT-T15 cells were embedded in agar on frosted slides, were rested for 1 h allowing repair to take place following trypsinisation. Islet and HIT-T15 cells were exposed for 30 min to the nitric oxide donor 3-morpholinosydnonimine (SIN-1) (100 μM), or to superoxide dismutase (SOD) (200 U/ml) for 30 min (HIT-T15 cells only) on agar on the slide, then assayed for DNA damage. Results represent the overall mean comet length ± S.E.M., *n* (in brackets) = 12–30 frames analysed for each treatment. Also shown incubation medium nitrite and nitrate concentrations (μM) ± S.E.M.

*$P < 0.05$, ****$P < 0.0001$ vs. control with no addition, +$P < 0.05$, and ++++$P < 0.0001$ vs. values obtained with SIN-1 (100 μM) alone. (A) = 3 independent experiments; (B) = 4 independent experiments; (C) = 2 independent experiments. Nitrite and nitrate determinations for (C) were performed independently with incubation of the cells in physiological buffer [25] (one experiment).

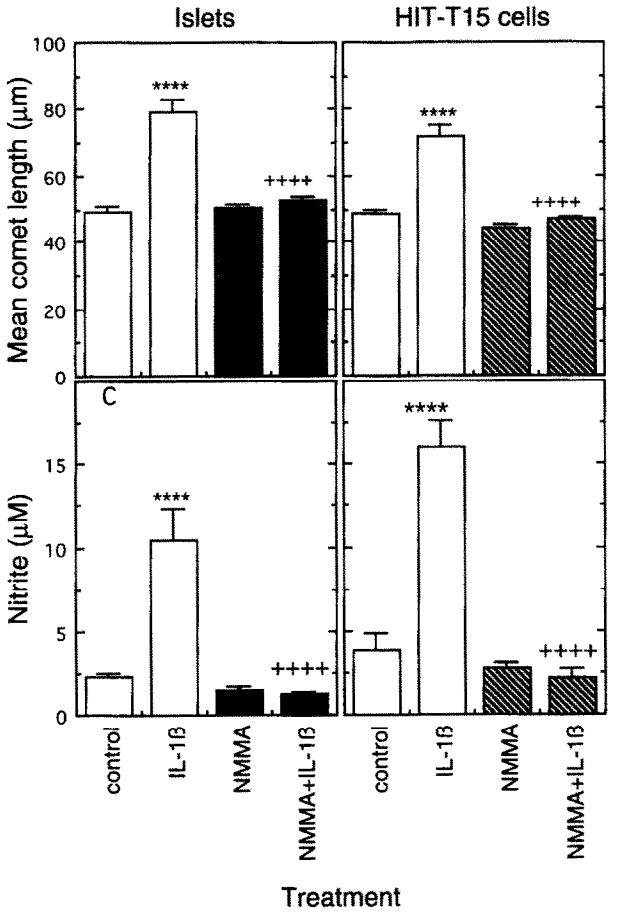

Fig. 1. DNA damage and nitric oxide levels following treatment of islets and HIT-T15 cells with interleukin-1β (18 h) in culture medium containing either L-arginine or NMMA. Following pre-culture in RPMI- 1640, groups of 100 islets or 4×10^5 HIT-T15 cells were incubated in RPMI-1640 medium prepared without arginine and supplemented with either L-arginine (1 mM) or *N*-nitro-mono-methyl-L-arginine (NMMA) (1 mM) and cultured for 12 h. Islets (A) and HIT-T15 cells (B) were then cultured for 18 h ± IL-1β (0.1 nM). Results represent the overall mean comet length (μm) ± S.E.M. from 4 separate experiments, n = 19–29 frames analysed for each treatment. DNA damage was observed in islets and in HIT-T15 cells only when nitric oxide was formed by IL-1β treatment (C,D). ****Represents values significantly increased vs. control $P < 0.0001$; and $^{++++}$represents values significantly less than obtained in arginine treatment conditions $P < 0.0001$ vs. IL-1β treatment by Student's t-test.

requires clean 3'-OH ends for polymerase activity. Hydrogen peroxide is a potent DNA strand-breaking agent, at very low doses (< 100 μM) in the comet assay [30], which should detect any type of nicking of supercoiled domains in the residual nuclear structure of the lysed cell [31]. 'Dirty' strand breaks may indeed be detected more readily in the comet assay because they are less rapidly repaired.

Use of the comet assay has enabled us to demonstrate, for the first time, that interleukin-1β-induced nitric oxide is sufficient to produce substantial DNA damage in normal islets and in an insulin containing cell line. In this study, nitric oxide damage seems to be related to cytokine induction of nitric oxide synthase since an arginine analogue (NMMA) prevents cytokine effects.

In a previous study, the inhibitory effects of IL-1β on insulin secretion and cyclic AMP production and stimulatory effects on cyclic GMP in islets were found to be mimicked by treatment of islets with SIN-1 (100 μM) for 30 min [32]. SIN-1 generates both nitric oxide and superoxide ions [33]. Nitric oxide may react with superoxide to form peroxynitrite ($ONOO^-$) [5], which is unstable at physiological pH, dismutating to form the highly reactive $OH^\cdot$ and $NO_2^\cdot$ radicals [7]. Removal of superoxide by superoxide dismutase extends the half-life of nitric oxide [34]. Neither the effects of SIN-1 on insulin secretion in islets (Cunningham et al., unpublished observations) nor DNA damage in HIT-T15 cells (Table I) were diminished in the presence of superoxide dismutase, which supports the idea that nitric oxide itself, rather than peroxynitrite, is mediating SIN-1 effects. We have also found significant DNA damage in islet cells following a 30 min treatment with the nitric oxide donor *S*-nitrosoglutathione, which is unlikely to generate superoxide. Recently, SIN-1-generated nitric oxide has been implicated in having both a neuroprotective and a neurodestructive effect depending on its oxidation–reduction status [7]. It is the neurodestructive effect which appears to be associated with peroxynitrite formation. Although hydroxyl radical formed from peroxynitrite would be expected to be a potent cytotoxic and strand-breaking agent, it appears that DNA strand breakage which we observe may be mediated largely by another mechanism. The nitrosative deamination of purines and pyrimidines described by Nguyen et al. [1] and Wink et al. [35] may be of greater importance.

Nitric oxide formation by SIN-1 was monitored by the production of nitrite, which is rapidly formed in aqueous solutions ($T_{1/2}$ = 6 s) by the oxidation of nitric oxide [36]. The measurement of nitrite as an indicator of nitric oxide formation has some limitations since nitric oxide is oxidised not only to nitrite but also to nitrate. We have measured both nitrite and nitrate formation from SIN-1 in the presence of superoxide dismutase. The smaller amount of nitrate formed in the presence of superoxide dismutase may reflect reduced formation of peroxynitrite and suggests that the failure of superoxide to affect strand break formation cannot be simply because superoxide dismutase is inactive in the system. The generation of nitric oxide from SIN-1 in cell-free medium appears to be enhanced in the presence of superoxide dismutase by blocking the nitric oxide scavenging effect of superoxide [34].

We have presented evidence that IL-1β-induced nitric oxide produces DNA damage in insulin-containing cells, the consequences for β-cell destruction and cell death remain to be examined. It is necessary to resolve the extent to which endogenously generated nitric oxide alone can lead to cell death or cell lysis, and whether the DNA damage we observe causes death directly or is a

signal for cells to die, for instance via programmed cell death.

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