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Repair of Cytokine-induced DNA Damage in Cultured Rat Islets of Langerhans

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Treatment of cultured rat pancreatic islets of Langerhans with the combined cytokines interleukin-1β (IL-1β), interferon γ (IFN γ) and tumour necrosis factor α (TNF α) leads to DNA damage including strand breakage. We have investigated the nature of this damage and its repairability. When islets are further incubated for 4 h in fresh medium, the level of cytokine-induced strand breakage remains constant. If the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (NMMA) is present during cytokine treatment, then strand breakage is prevented. If NMMA is added following, rather than during, the cytokine treatment and islets are incubated for 4 h, further nitric oxide synthesis is prevented and most cytokine-induced strand breaks are no longer seen. To investigate DNA repair following cytokine treatment, cells were transferred to fresh medium and incubated for 4 h in the presence of hydroxyurea (HU) and 1-β-D-arabinosyl cytosine (AraC), as inhibitors of strand rejoining. In the presence of these inhibitors there was an accumulation of strand breaks that would otherwise have been repaired. However, when further nitric oxide synthesis was inhibited by NMMA, significantly less additional strand breakage was seen in the presence of HU and AraC. We interpret this, as indicating that excision repair of previously induced base damage did not contribute significantly to strand breakage. Levels of oxidised purines, as indicated by formamidopyrimidine glycosylase (Fpg) sensitive sites, were not increased in cytokine-treated islets. We conclude that in these primary insulin-secreting cells: (a) the DNA damage induced by an 18 h cytokine treatment is prevented by an inhibitor of nitric oxide synthase, (b) much of the damage is in the form of apparent strand breaks rather than altered bases such as oxidised purines, (c) substantial repair is ongoing during the cytokine treatment and this repair is not inhibited in the presence of nitric oxide.

Keywords: Comet assay; DNA repair; Nitric oxide; Hydroxyurea; 1-β-D-arabinosyl cytosine; N^G-monomethyl-L-arginine

Abbreviations: 8-OHG, 8-hydroxyguanine (7,8-dihydro-8-oxoguanine); AraC, 1-beta-D-arabinofuranosyl cytosine; Fpg, formamidopyrimidine glycosylase; HU, hydroxyurea; IFNγ, interferon γ; IL-1β, interleukin-1β; MEM, minimum essential medium with Earle's salts; NMMA, N^G-monomethyl-L-arginine monoacetate; TNFα, tumour necrosis factor α

INTRODUCTION

Treatment of pancreatic islets of Langerhans by the proinflammatory cytokine interleukin-1β (IL-1β) causes generation of nitric oxide and/or reactive oxygen species, leading to inhibition of insulin secretion.[1–6] We and others have previously shown that IL-1β on its own caused DNA strand breakage in rat islets and in a β-cell line.[7] In addition, a combination of proinflammatory cytokines induced strand breaks in rat[8] and human islets of Langerhans[9] and in immunopurified primary rat β-cells.[10] Damage was prevented completely (over shorter periods[9]), or partially (over longer periods[8]), by co-incubation with an inhibitor of nitric oxide synthase, suggesting nitric oxide involvement in the DNA damaging activity. Since low levels of defence mechanisms against reactive nitrogen and oxygen species could contribute to β-cell death,[11,12] we have examined the extent to which islets can repair the DNA strand breaks induced following treatment with cytokines.

DNA damage induced by cytokines may include both strand breaks and damaged bases. The types of damage are likely to depend on the extent to which cytokines induce reactive oxygen species in addition

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to nitric oxide. Nitric oxide may react with oxygen to form the DNA-damaging species N_2O_3.[13] Combined formation of nitric oxide and superoxide should lead to formation of the potent DNA-damaging species peroxynitrite,[14,15] and the damaging activity of nitric oxide is often largely ascribed to this molecule. Rabinovitch and Suarez-Pinson[16] have suggested that cytokines can induce formation of peroxynitrite. However, we and others have found that inhibition of insulin secretion by cytokines resembles inhibition by a "pure" nitric oxide donor rather than by peroxynitrite or a mixed nitric oxide/superoxide donor.[9,17]

In the experiments described here, we have used the comet assay (single cell gel electrophoresis)[7,18] to study cytokine-induced DNA damage and repair in primary insulin-secreting β-cells. The comet assay will detect both true strand breaks, and some types of alkali-labile lesions, including abasic sites. It can also be used to detect specific types of damaged base, through incorporating an incubation step with a variety of purified repair enzymes.[19,20] The enzyme forms a strand break in the vicinity of the altered base, as the first stage in the excision repair process. One such base, 7,8-dihydro-8-oxoguanine (8-OHG), is commonly regarded as an indicator of oxidative damage.[21] This lesion is probably not formed by nitric oxide alone, but in some cases may be formed by combined treatment with nitric oxide and reactive oxygen species. De Rojas-Walker *et al.*[22] found increased levels of 8-OHG in lipopolysaccharide-activated macrophages, though Gal *et al.*[23] saw no increase in spleens of tumour-bearing mice. 8-OHG is removed by a DNA glycosylase, with 8-oxoguanine glycosylase (Ogg1) being the mammalian equivalent of the bacterial formamidopyrimidine glycosylase (Fpg). These enzymes recognise and excise oxidised and ring-opened purines including 8-OHG.[24] We have used Fpg in conjunction with the comet assay to look for cytokine induction of 8-OHG or other oxidised purines in cultured rat islets.

Damage to islet DNA induced by cytokines can be partially[8] or completely[9] prevented by carrying out the cytokine treatment in arginine free medium supplemented with a nitric oxide synthase inhibitor. Corbett and McDaniel[25] have gone further, and shown that inhibition of insulin secretion by IL-1β can still be reversed when the inhibitor is added to islets some time after the cytokine treatment has been initiated. Such an experiment suggests a way of studying repairability of cytokine-induced damage. Islets can be treated with cytokines until induction of DNA damage is seen. If an inhibitor of nitric oxide synthase is added at this stage, no new DNA damage will be induced by nitric oxide, and it should be possible to observe repair of the nitric oxide mediated damage already present. If no further DNA damage is created in such an experiment, the exact effect will depend on the type of damage. Direct strand breaks (and alkali-labile sites) should decrease fairly rapidly as they are repaired. On the other hand, strand breaks will continue to be generated from the excision repair process acting on bases already damaged (including 8-OHG), even though no new damage is occurring. If strand rejoining is blocked, an accumulation of these excision-derived breaks will be seen. By inhibiting the cell's own repair processes, we can reveal as wide a range of damage as possible.

In our experiments, we have used a combined treatment with hydroxyurea (HU) and cytosine arabinoside (1-beta-D-arabinofuranosyl cytosine, AraC) to block the resynthesis required to allow DNA strand break rejoining. HU is an inhibitor of ribonucleotide reductase, the rate-limiting enzyme that catalyses the reduction of ribonucleotides to the corresponding deoxyribonucleotides,[26] so that it blocks *de novo* synthesis of the deoxyribonucleotide precursors of DNA synthesis. AraC is phosphorylated via nucleoside scavenging pathways, and incorporated into DNA where it may act to slow DNA synthesis and strand rejoining.[27,28] On their own, neither is particularly effective in mammalian cells. HU does not block scavenging pathways of precursor synthesis and, without blocking of *de novo* synthesis, insufficient AraC is incorporated to be effective. Together, however, they are as effective as the specific polymerase inhibitor aphidicolin.[29,30] Although these inhibitors would be expected to inhibit rejoining less strongly following base excision than following nucleotide excision, there is empirical evidence that they do affect base excision (e.g. Ref. [31]). This point is considered in detail in the "Discussion" section.

Nitric oxide has been shown to inhibit the activity of enzymes involved in DNA repair and replication, including the bacterial Fpg[32] and mammalian Ogg1[33] enzymes involved in repair of oxidised purines. We looked for an effect of cytokine treatment on DNA sites sensitive to the bacterial enzyme, Fpg. Other enzyme targets for nitric oxide include ligase,[34] and ribonucleotide reductase.[35] Inhibition of ribonucleotide reductase will inhibit DNA synthesis and should also cause accumulation of the DNA strand breaks by delaying strand rejoining. It is possible to substitute for ribonucleotide reductase by supplementing medium with deoxyribonucleosides, which are incorporated into dNTPs and DNA by salvage pathways.[36,37] As an additional speculative experiment, we also investigated whether the occurrence of DNA strand breaks following cytokine treatment could be corrected by deoxyribonucleosides.

MATERIALS AND METHODS

Materials

The cytokines (IL-1β, interferon γ (IFNγ) and tumour necrosis factor α (TNFα) were obtained from AMS Biotechnology, Abingdon, UK. Accutase™ was from TCS Cell Works, Buckingham, UK. NuSieve low melting point agarose was from FMC Bioproducts, Rockland, MD. Fpg enzyme was the kind gift of Dr A.R. Collins (Rowett Institute, Aberdeen, Scotland). Wistar rats were obtained from Charles Rivers (Sandwich, Kent, UK). Islet culture media were from Gibco/Invitrogen (Paisley, UK) and other chemicals from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise indicated.

Isolation and Treatment of Pancreatic Islets of Langerhans

Islets from 55 to 60-day-old Wistar rats were isolated by ductal injection of collagenase (Sigma type XI),[38] 1.1 mg/ml in ice cold Gey and Gey buffer.[39] Islets were pre-cultured for 48 h before treatment, under a humidified atmosphere of 95% air/5% CO_2 at 37°C in 35 mm multiwell plates in glucose-free RPMI 1640 medium supplemented with D-glucose (5.5 mmol/l) foetal calf serum (5% v/v), L-glutamine (2 mmol/l) penicillin (100 U/ml) and streptomycin (100 μg/ml). In each experiment, islets from 3 to 4 animals were pooled and then split for individual treatments. This ensured that each treatment involved the same population of islets and that there were sufficient cells for the comet assays. After the initial incubation, islets were re-picked into 35 mm Petri dishes and treated with cytokines IL-1β (20 U/ml) + IFNγ (5 U/ml) + TNFα (100 U/ml) for a further 18 h. After treatment, fresh medium was added for a further 4 h incubation in the presence or absence of the arginine analogue and nitric oxide synthase inhibitor N^G-monomethyl-L-arginine monoacetate (NMMA, 1 mmol/l). In some experiments, HU (10 mmol/l) and 1-β-D-arabinosyl cytosine (AraC, 10 μmol/l) were included in the medium, to prevent strand rejoining. Incubations with NMMA were performed using arginine free RPMI 1640.

Islet Disaggregation

Following treatment, islets were disaggregated into single cell suspensions using minimal digestion with 1 ml of Accutase solution, incubating for 12–13 min at 37°C. Once a satisfactory separation of islet mass into single cells was obtained, cells were rinsed with RPMI 1640 medium supplemented as indicated above, the cell suspension was centrifuged at 200*g* for 5 min and the supernatant was discarded. Dissociation into single cells was carried out gently, in order not to introduce additional DNA strand breakage.

Comet Assay

The comet assay was performed by a modified protocol of Singh *et al.*[18] as described previously,[40] but using standard microscope slides on which a layer of agarose had been predried.[41] Equal volumes of 1.4% NuSieve (FMC Bioproducts, Rockland, MD) low melting point agarose solution and cell suspension in RPMI 1640 were mixed, 50 μl of the mixture containing approximately 5×10^4 cells was added on to a coated microscope slide, and a coverslip was added to spread the agarose. Coverslips were removed and the slides were placed in lysis mixture (2.5 mol/l NaCl, 100 mmol/l EDTA–Na_2, 10 mmol/l Tris, 200 mmol/l NaOH, 1% v/v Triton X-100, 10% dimethylsulphoxide, pH10) at 4°C for at least 1 h. To detect strand breaks (or alkali-labile sites), slides were placed in alkaline buffer (300 mmol/l NaOH, 1 mmol/l EDTA–Na_2, pH > 13), incubated for 40 min at 15°C, and subjected to electrophoresis (20 V or 0.8 V/cm, 24 min). To detect Fpg-sensitive sites, slides were taken from lysis mixture, rinsed three times with enzyme buffer (40 mmol/l HEPES, 100 mmol/l KCl, 0.5 mmol/l EDTA–Na_2, 0.2 mg/ml bovine serum albumin (Fraction V), pH 8), and the surface of the agarose blotted dry. Fifty microlitres of Fpg enzyme (4 μl/ml of stock extract as supplied) in enzyme buffer was added to the surface of the agarose, a coverslip was applied, and the slide was incubated at 37°C for 30 min. Sites of oxidised purines will be converted to strand breaks by this procedure. The net number of Fpg-sensitive lesions is seen as the additional strand breaks in a slide treated with Fpg enzyme over a control slide incubated with buffer and without Fpg enzyme. The slides were transferred to alkaline buffer, and subjected to immediate electrophoresis (20 V, 24 min), with no unwinding period. The concentration of enzyme used was approximately 12-fold times higher than the recommended level, so that some non-specific incision may have occurred. However, in trials, lowering the concentration of enzyme did not improve the specificity of detection of induced Fpg-sensitive sites.

Following electrophoresis, the slides were rinsed with Tris–EDTA buffer pH 7.5, stained with ethidium bromide (20 μg/ml), and scored under a fluorescent microscope with a ×10 objective, using the Casys system (Synoptics, Cambridge, UK). Comet length was taken as a measure of DNA strand break frequency. The alternative parameters, tail moment and % DNA in tail, were also scored. Normally all these parameters give similar results, but in order to compare differences between differences, it is desirable to use a parameter that

increases linearly with damage. We have previously shown this for comet length.[42] At least 50 nuclei on two slides were scored for each treatment.

Insulin Secretion

The radioimmunoassay for rat insulin has been described previously.[9] Islets were removed from culture and pre-incubated for 1 h at 37°C in physiological buffer[39] containing 2 mmol/l glucose. The insulin secretory response was evaluated by incubating groups of 5 islets in 600 μl of buffer containing 2 and 20 mmol/l glucose for an additional 1 h. A 500 μl aliquot was removed, diluted and assayed for secreted insulin. The insulin contained within the islets was extracted with the addition of 100 μl of an acid-ethanol solution (23/7/0.45 v/v/v ethanol/water/HCl) and left overnight at 4°C. Sample extracts were diluted and assayed for insulin. Insulin release was expressed both in ng/islet/h and as a percentage of the total insulin content of the islets.

Nitrite and Nitrate Assays

For experiments to determine nitrate and nitrite formation, the islets were cultured in nitrate-free minimum essential medium with Earle's salts (MEM) supplemented with D-glucose (5.5 mmol/l), foetal calf serum (5% v/v), L-glutamine (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 μg/ml) (all from Gibco BLR, Paisley, UK). RPMI 1640 cannot be used in this case, because it contains 427 μmol/l $Ca(NO_3)_2 \times 4H_2O$. Islets were treated for 18 h with the combination of cytokines and concentrations as described above but in MEM medium. Nitrate was converted to nitrite using nitrate reductase (625 U/ml) and β-NADPH (151 μmol/l), both dissolved in 40 mmol/l Tris buffer, pH 7.6, incubating at 20°C for 3 h. The converted nitrate plus nitrite concentration was then measured by addition of Griess reagent and reading absorbance at 540 nm.[43] Nitrite alone was measured by omitting the enzyme treatment, and after 5 min incubation with Griess reagent, measuring absorbance at the same wavelength. Nitrate and nitrite calibration standards (range 2–32 μmol/l) were prepared in Tris buffer pH 7.6. In some experiments, where islets were incubated in RPMI 1640, nitrite formation alone was assayed as an indicator of nitric oxide synthase activity.

Statistical Analysis

Median comet length was determined for each treatment in each experiment, since this is less influenced than the mean by rare heavily damaged cells. The means of these median values were then compared by a paired Student *t*-test. This approach is more conservative than the non-parametric Mann–Whitney *U* test (which we also performed, pooling the data from repeat experiments), since the critical question is whether an apparent effect is consistent between experiments.

RESULTS

Repair of DNA Strand Breaks

Cells from rat islets treated for 18 h with a combination of cytokines IL-1β (20 U/ml), IFNγ (5 U/ml) and TNFα (100 U/ml) showed a significant increase in DNA damage (strand breaks) as indicated by the comet assay (Fig. 1A). We refer to the DNA damage seen as strand breaks, although some of it could represent alkali-labile sites. Formation of strand breaks was almost completely prevented by co-incubation with the arginine analogue and nitric oxide synthase inhibitor NMMA, suggesting that the DNA damage was nitric oxide mediated (Fig. 1A). When islets were treated with cytokines (without NMMA), then transferred for 4 h to fresh arginine-free medium containing NMMA in order to inhibit further nitric oxide formation, the number of strand breaks was substantially reduced, whereas when islets were transferred to fresh medium and cultured for 4 h in the absence of NMMA, the number of strand breaks showed no reduction (Fig. 1B). This would be expected, since removal of cytokines will not cause immediate downregulation or inactivation of nitric oxide synthase. We conclude that substantial repair of cytokine-induced DNA damage is seen when continued production of nitric oxide is blocked. When cytokine-treated islets were allowed to recover for a full 24 h in the presence or absence of NMMA, both groups showed fewer strand breaks than the 18 h cytokine-treated group, but the effect of NMMA persisted (Fig. 1B). The amount of damage remaining at the end of the 24 h recovery period in the presence of NMMA was as low as in the 18 h control, indicating nearly complete repair of strand breaks following termination of nitric oxide generation.

In separate experiments, when islets were incubated for 18 h with cytokines in nitrate-free MEM culture medium, nitrate (27.9 ± 9.4 pmol/islet) and nitrite (39.7 ± 9.8 pmol/islet) were significantly higher compared to the respective controls (7.1 ± 4.4 and 9.0 ± 4.7 pmol/islet). The relative proportions were 36% nitrate and 64% nitrite. Although strand breaks were reduced at the end of the 24 h recovery period following cytokine treatment, insulin secretion in response to glucose was not fully restored (Table I), particularly when NMMA was present.

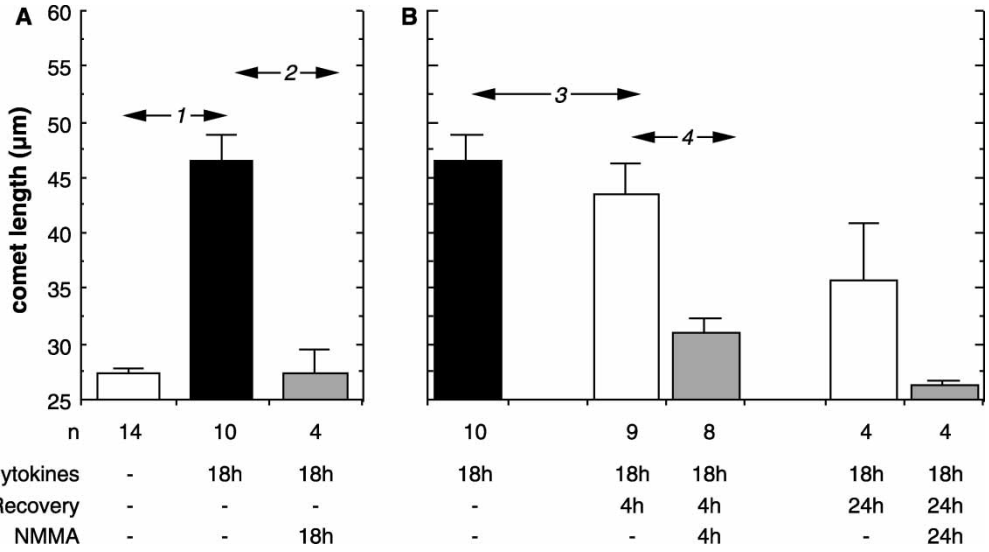

FIGURE 1 Cytokine-induced DNA strand breakage in cultured rat islets of Langerhans. DNA strand breakage was determined as an increase in comet length, using the standard comet assay. A minimum of 50 nuclei were analysed per treatment per experiment. Data are plotted as averages ± SEM of median comet lengths from individual experiments. Comparisons are by paired Student *t*-test. (A) Wistar rat islets cultured with cytokines for 18 h in the presence or absence of NMMA (1 mM in RPMI 1640 arginine-free medium): (1) 18 h cytokine treatment vs. untreated control, $P < 0.0001$, $n = 10$; (2) cytokine treatment vs. cytokines + NMMA, $P < 0.002$, $n = 4$. Figures present all data for a given treatment, but comparisons by paired t-test are confined to cases where both treatments were performed within the same experiment. (B) Following 18 h incubation with cytokines, islets were transferred to medium without cytokines for a 4 or 24 h recovery period in the presence or absence of NMMA. (3) 18 h cytokine treatment vs. 18 h cytokine treatment + 4 h fresh medium, $P > 0.11$, $n = 5$, NS; (4) cytokines + 4 h fresh medium vs. cytokines + 4 h NMMA, $P < 0.03$, $n = 4$.

Effect of Inhibition of Strand Rejoining

The process of excision of damaged bases will lead to additional DNA strand breaks being formed, before repair can be completed by strand rejoining. Inhibition of strand rejoining would be expected to lead to an accumulation of these additional excision-induced strand breaks, even without induction of new DNA base damage. In contrast, although inhibition of rejoining should cause directly induced strand breaks to persist, additional breaks would not be expected to form. To distinguish between these possibilities, islets were treated with cytokines and incubated with HU and AraC for 4 h. Under these conditions nitric oxide would continue to be synthesised, and new damage would be expected to occur. Significantly more strand breaks were seen ($P < 0.007$, Fig. 2A) in the presence of HU and AraC. When NMMA was present following cytokine treatment, little new damage would have been expected to form, although excision of preexisting breaks should occur. Under these conditions, HU and AraC gave a significantly smaller ($P < 0.003$) increase in the number of strand breaks observed. This suggested that: (a) little excision of damaged bases was occurring subsequent to the cytokine treatment, (b) the predominant damage induced by cytokines consisted of direct strand breaks, and (c) a number of the strand breaks induced by nitric oxide were in fact being repaired during the treatment period, so that the breaks seen at the end of the 18 h cytokine

TABLE I Inhibition of insulin secretion by cytokines

	Insulin secretion			
	2 mM glucose (ng/islet/h)	20 mM glucose (ng/islet/h)	2 mM glucose (% total islet insulin content)	20 mM glucose (% total islet insulin content)
Control 18 h	0.31 ± 0.08	1.24 ± 0.21*	0.85 ± 0.24	2.70 ± 0.30*
Cytokines 18 h	0.59 ± 0.19	0.33 ± 0.15**	1.72 ± 0.46	0.95 ± 0.35**
Control 42 h	0.40 ± 0.19	1.27 ± 0.29*	1.24 ± 0.67	4.19 ± 1.25*
Cytokines 42 h	0.81 ± 0.38	0.68 ± 0.30**	2.31 ± 1.51	2.47 ± 2.17**
18 h cytokines + 24 h with NMMA	0.52 ± 0.2	0.62 ± 0.07	3.02 ± 0.94	3.31 ± 0.40
18 h cytokines + 24 h without NMMA	0.41 ± 0.16	0.86 ± 0.10*	1.27 ± 0.63	2.80 ± 0.70

*$P < 0.01$ vs. 2 mM glucose control. **$P < 0.01$ vs. control 20 mM glucose, all by ANOVA. Islets were treated with cytokines as shown, followed by a recovery period in arginine-free medium with or without NMMA (1 mM). Insulin secretion by islets incubated in 2 or 20 mM was determined as described in "Methods" section and expressed both as ng of insulin secreted/islet/h and also as a percentage of the total insulin content of the islets. Results are shown as Mean ± SEM from four separate experiments.

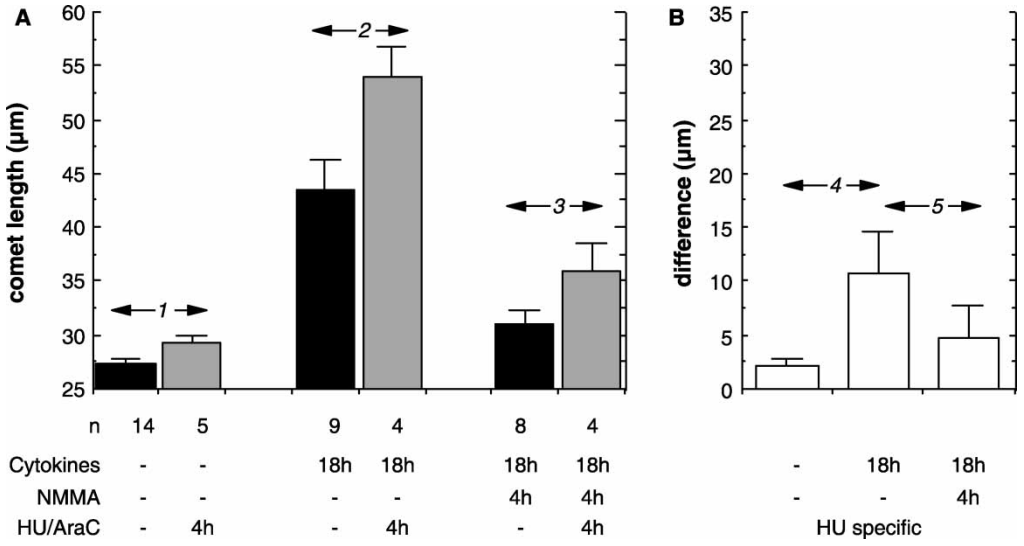

FIGURE 2 Effect of inhibition of strand rejoining on the level of DNA strand breakage in cytokine-treated islets. (A) Rat islets were treated for 18 h with or without cytokines. Cytokines were removed and the islets were further incubated for a further 4 h with or without NMMA (1 mM) and/or HU/AraC (final concentration 10 mM/10 μM). DNA strand breakage was determined as an increase in comet length, using the standard comet assay. A minimum of 50 nuclei were analysed per treatment per experiment. Data are plotted as averages of median comet lengths from individual experiments ±SEM. Figures present all data for a given treatment, but comparisons by paired *t*-test are confined to cases where both treatments were performed within the same experiment. (1) control vs. control + HU/AraC $P > 0.85$, $n = 4$, NS; (2) cytokines 18 h + 4 h fresh medium vs. cytokines 18 h + 4 h HU/AraC, $P < 0.007$, $n = 4$; (3) cytokines 18 h + 4 h NMMA vs. cytokines 18 h + 4 h NMMA and HU/AraC, $P > 0.08$, $n = 4$, NS. (B) The same data presented as the increase in comet length due to the presence of HU/AraC. (4) HU/AraC effect in control vs. effect following cytokine treatment, $P < 0.006$, $n = 3$; (5) HU/AraC effect following cytokine treatment in the absence vs. presence of NMMA, $P < 0.003$, $n = 4$.

treatment represented only a proportion of the total damage that had been induced. Comet length has been chosen here as the parameter to indicate damage, since it increases approximately linearly (over a range of 30–70 μm) with dose.[42]

In addition to their inhibitory effects on DNA synthesis, HU is a potential nitric oxide donor,[44] and AraC can induce oxidative stress in neurones.[45] Therefore, as an additional control, islets cultured for 18 h without cytokines were further incubated for 4 h with HU/AraC. Only slight formation of strand breaks (Fig. 2A) was observed, suggesting that the potential DNA-damaging effects of these agents made a minimal contribution under the conditions of the experiment. The differences in comet length in the presence vs. absence of HU/AraC are shown in Fig. 2B.

It occurred to us that some of the strand breakage seen following cytokine treatment might just possibly be the result of nitric oxide inhibition of ribonucleotide reductase,[35] leading to delayed strand rejoining. In this case, addition of exogenous deoxyribonucleosides should bypass ribonucleotide reductase and allow formation of dNTPs via scavenging pathways. However, addition of the four deoxyribonucleosides at 10 μmol/l did not give a significant reduction in the level of strand breakage induced by 18 h combined cytokine treatment (two experiments, median comet length without deoxyribonucleosides 43.1 μm, with deoxyribonucleosides 37.1 μm, $P > 0.6$).

Formation of Oxidative DNA Damage as Fpg-sensitive Lesions

We next tested for evidence of cytokine-induced formation of oxidised purines. However, rat islets cultured even in the absence of cytokines showed substantial oxidative DNA damage, detected as Fpg-sensitive lesions in the modified protocol of the Comet assay (Fig. 3A). Oxidative damage was also seen in cytokine-treated islets co-incubated with NMMA (Fig. 3A). This may have included some non-specific damage, through using a high concentration of enzyme in the assay. Trial experiments, however, showed no improvement in specificity of the assay with lower concentrations of enzyme. In separate western blotting experiments islets in culture showed upregulation of haem oxygenase (data not shown), suggesting a degree of oxidative stress. Cytokine treatment gave no increase in percent DNA in tail due to Fpg-sensitive sites.

DISCUSSION

The nature of the β-cell DNA damage induced by cytokines and associated free radicals is complex. Nitric oxide itself can damage DNA,[46] perhaps via N_2O_3,[13] and can react with superoxide to form peroxynitrite.[15] It can also inhibit enzymes associated with DNA repair [33] and strand rejoining,[34,35] though inhibition of ribonucleotide

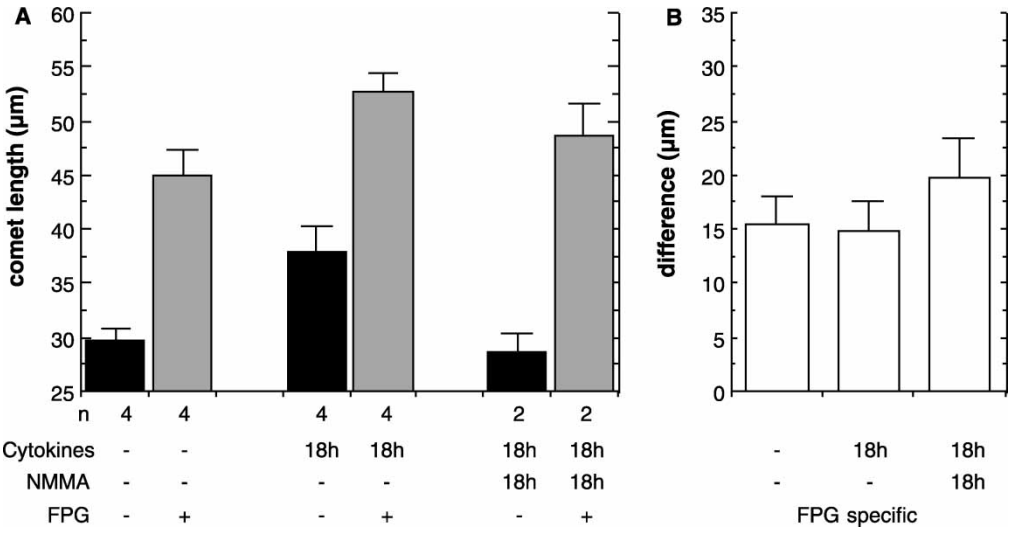

FIGURE 3 Fpg-sensitive sites in cytokine-treated rat islets. (A) Wistar rat islets were cultured with cytokines for 18 h, without cytokines, or with cytokines and NMMA. Using a modified protocol of the comet assay, slides were incubated with Fpg enzyme or a buffer control and damage determined as an increase in comet length. Mean ± SEM of median comet length. (B) The same data presented as the increase in Fpg-specific strand breaks.

reductase does not appear to contribute to the strand breakage seen in the present experiments.

Increased formation of 8-OHG *in vivo* is not consistently found under conditions of induction of nitric oxide synthase.[22,23] In some studies, mononuclear cells from patients with insulin-dependent and non-insulin dependent diabetes have shown increased formation of oxidative damage[47–50] or strand breakage.[51] Other studies, e.g.[52–55] have been more equivocal or negative. Fpg-sensitive lesions have also been induced in cholangiocarcinoma cells incubated with cytokines.[56] In our experiments, there was a high apparent background level of 8-OHG during islet culture, and no increase was seen with cytokine treatment.

The comet assay has a specific disadvantage that Fpg-sensitive sites must be detected against a background of cytokine-induced direct strand breaks. However, the method requires far less material than other approaches, and is more resistant to accidental oxidation of DNA. Reliable detection of 8-OHG by electrochemistry or immunoassay[57] would have required more starting material than was feasible in our experiments (perhaps islets from up to 100 animals per treatment (Arimoto-Kobayashi, personal communication).

Rather than base damage, the major cytokine-induced lesions in DNA appear to be direct DNA strand breaks, which would not be distinguished from alkali-labile sites in our experiments. Potential unstable types of base damage include xanthine, formed by deamination of guanine[58] and 8-nitroguanine,[59] though it is not clear whether the latter plays a significant role.[60,61]

The observations with inhibition of strand break rejoining are informative. Accumulation of strand breaks is seen when rejoining is inhibited in the presence of nitric oxide, suggesting that much nitric oxide damage may be relatively rapidly repaired, and the damage seen after prolonged incubation will be likely to include a residue of hard-to-repair damage. The lack of strand break accumulation when rejoining is inhibited in the absence of nitric oxide tends to confirm that alkali-stable base damage may not constitute a significant proportion of overall cytokine-induced damage. Our results would be consistent with the major cytokine damage induced in islet cells consisting of DNA strand breaks and apurinic sites arising from deamination. Although nitric oxide can inhibit repair of several types of DNA damage, our results suggest that substantial repair is occurring in the presence of nitric oxide.

Such a conclusion depends on HU and AraC being effective inhibitors of base excision repair. It is likely that they will inhibit base excision repair of oxidative damage less effectively than nucleotide excision repair of bulky lesions. Base excision resynthesis is mainly performed by DNA polymerase β, which is less sensitive to AraC than polymerase α,[31,62–64] and much base excision repair involves replacement of only a single nucleotide.[65] However, polymerase β can also perform "long patch" resynthesis.[66,67] Other polymerases substitute for polymerase β in this process, so that polymerase β null cells are not necessarily hypersensitive to oxidative damage,[68,69] and may show normal removal of 8-oxoguanine.[70] In practice, DNA synthesis inhibitors do inhibit base excision repair. Aphidicolin has been shown to affect base excision repair of adenine opposite 8-oxoguanine.[71] AraC affects base excision repair of ionising radiation damage,[31,72] and increases hydrogen peroxide induced micronucleus formation.[73]

It is not entirely clear why AraC is effective as an inhibitor of base excision break rejoining. Unlike a dideoxynucleotide, it is not an especially effective terminator of chain elongation (e.g. Ref. [62]). AraC may compete with dCTP for binding to polymerase,[63,64] but only moderately in the case of DNA polymerase β. However, since excision breaks are normally only open transiently, any delay in rejoining should give a substantial increase in the number of breaks seen at a given time.

Although we observe repair of DNA strand breaks in these experiments, the cytokine + NMMA treatment is too damaging to allow full recovery of islet secretory function (cf. Ref. [8]). IL-1β on its own will damage rat islet cell DNA,[7] but to a lesser extent than a combination of IL-1β + TNFα + IFNγ[9] (Rosales Hernandez, unpublished). Exposure of rat β-cells to the combination of IL1β + IFNγ has also been shown to cause greater induction of defence/repair genes than exposure to IL-1β alone.[74] Corbett and McDaniel[25] have shown that the inhibitory effect produced by IL-1β on β-cell insulin secretion can be reversed after 18 h, by incubation with NMMA. IFNγ + TNFα treated islets, allowed to recover for 7 days, remained unresponsive to glucose.[75] In our experiments we could not observe restored responsiveness to glucose in islets treated with three cytokines and allowed to recover for 24 h with NMMA, suggesting more severe damage to β-cells. Cytokines such as IL-1β and TNFα are certainly released from cells of the immune system in the inflammatory process initiated against β-cells (Refs. [76,77] for reviews) therefore the β-cell insulin secretory response may be irreversibly damaged. Our results, however, indicate that under the same conditions, cytokine-induced DNA strand breaks are extensively repaired, with repair successfully occurring in the presence of nitric oxide.

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