Comparison of serum F2 isoprostane levels in diabetic patients and diabetic patients infected with *Burkholderia pseudomallei*

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ABSTRACT

Introduction: Oxidative stress can occur in sepsis and infection, when overproduction of free radicals is not countered by the host antioxidant system, leading to impairment of host cellular functions. Various disease states are accompanied by the accumulation of 15-F_{2t}-IsoP in biological fluids. These isoprostanes are considered as markers of oxidative stress, and inflammation and inflammatory mediators.

Methods: We measured total serum 15-F_{2t}-IsoP levels by the immunoassay method in healthy adults, otherwise healthy patients with diabetes mellitus and diabetic patients infected with *Burkholderia pseudomallei (B. pseudomallei)*.

Results: The highest mean value of 4,343.6 pg/ml of 15-F2t-IsoP was found in the diabetic-melioidosis patients in comparison with the uninfected diabetic patients and the normal controls. Uninfected diabetic patients had significantly higher levels than the control subjects (p-value is less than 0.001), but lower than the diabetic-melioidosis patients (p-value is less than 0.001). The main finding of the present study was an eight-times higher median circulating total IsoPs levels in diabetic patients infected with *B. pseudomallei* when compared with the levels in control subjects.

Conclusion: The oxidative stress theory proposes that severe sepsis leads to activation of neutrophils and macrophages which subsequently release reactive oxygen-free radicals that may result in lipid peroxidation of endothelial and epithelial cell membrane phospholipids. This chain reaction results in increased levels of isoprostanes, which are thought to contribute to much of the end-stage tissue damage seen in serious infections, such as melioidosis. We believe that this is the first report linking *in vivo* oxidative stress status and diabetic patients infected with *B. pseudomallei*.

Keywords: *Burkholderia pseudomallei*, diabetes mellitus, F2 isoprostanes, immunoassay

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INTRODUCTION

Potential biotoxic products produced by oxidative stress can be markedly enhanced under conditions of exogenous oxidative stress, particularly by phagocytes as a means of host defence against invading microorganisms.[1] Lipid peroxidation is a central feature of oxidative stress, and one of its secondary end-products is a group of prostaglandin (PG)F_2-like products termed "F_2-isoprostanes." Isoprostanes are a complex family of compounds, produced from arachidonic acid via a free-radical-catalysed mechanism, which are formed *in situ* in phospholipids. Once released from cell membranes by phospholipases, isoprostanes circulate in the plasma and biological fluids in free forms. They can be quantified as reliable markers of lipid peroxidation resulting from oxidative stress. Among the isoprostanes, the 15-F_{2t}-IsoP has been shown to be stable, biologically active and produced in abundance during oxidative stress.[2] Isoprostanes were formerly named according to the prostaglandin $F_{2\alpha}$ chemical structure, but in 1997, Taber et al proposed a new nomenclature. The old name 8-iso-prostaglandin $F_{2\alpha}$, or 8-epi-prostaglandin $F_{2\alpha}$, was renamed as 15-F_{2t}-IsoP.[3]

Significant levels of 15-F_{2t}-IsoP are found in normal human biological fluids and tissues, indicating an ongoing lipid peroxidation that is incompletely suppressed by antioxidant defences. Therefore, enhanced oxidative stress associated with various factors, such as human pulmonary disease, chronic smoking, allergen-induced asthma, diabetes mellitus, bacterial infection and sepsis, will lead to increased free radical production, including 15-F_{2t}-IsoP.[2] Studies examining the utility of quantifying IsoPs as an index of oxidative stress in association with human disease, particularly in pulmonary disorders and increased levels have been shown in acute respiratory distress disease (ARDS) and acute lung injury (ALI), among others.[4] These provide evidence that oxidative stress is increased in a number of pulmonary disorders and that IsoP generation may play a role in the pathophysiology of these diseases. Quantitation of oxidative stress might provide insight into the mechanisms of lung injury. Such data might, in individual patients, provide prognostic information, or identify a subset of patients who could possibly benefit from antioxidant therapy.[4] Previously, we had reported

that in 50 patients with septicaemia due to *Burkholderia pseudomallei (B. pseudomallei)*, the mortality was 73% if the respiratory system was affected.[5] In another previous report, six patients with fatal septicaemic melioidosis had rapid development and progression of respiratory failure due to ALI and/or ARDS;[6] We had hypothesised that the rapidity of the onset of ARDS may have been due to the activation of polymorpholeucocytes with the release of bacteria and toxic-free radicals and other products of cellular degradation, leading to massive endothelial damage and lung injury.[6] Approximately 50% of adult melioidosis patients have diabetes mellitus. As both hyperglycaemia and hyperinsulinaemia increase free radical production, diabetes mellitus increases the burden of oxidative stress in patients with melioidosis. Thus, in the present study, we undertook to measure the levels of 15-F_{2t}-IsoP in three groups of subjects to assess the state of oxidative stress, and whether increased levels of IsoPs have a role in the pathophysiology in patients with diabetes mellitus and sepsis, particularly when they are infected with *B. pseudomallei*. These groups are: (a) normal healthy adults; (b) patients with diabetes mellitus who were otherwise healthy; and (c) melioidosis patients who also had diabetes mellitus.

METHODS

Blood samples were collected into plain tubes from the following groups of people: (a) 25 healthy adults who were blood donors at the hospital; (b) 25 patients with diabetes mellitus attending the outpatient diabetic clinic and were otherwise healthy; and (c) 25 patients admitted to the hospital and undergoing treatment for melioidosis (age-, sex- and race-matched). All control subjects were in good health and all participants gave informed consent. Samples were immediately spun at 500 g for ten minutes and the serum was transferred into microcentrifuge tubes and stored at -70°C.

The whole procedure of 15-F_{2t}-IsoP determination was performed according to the manufacturer's recommendations. (Direct 8-iso-Prostaglandin $F_{2\alpha}$ Enzyme Immunoassay Kit. Catalog no. 900-091, Assay Designs, Ann Arbor, MI, USA). Hydrolysis of the esterified bonds between phospholipids and 15-F_{2t}-IsoP, was carried out in order to measure the total (free + esterified) isoprostane levels. 800 µL of serum samples were treated with 200 µL of 10 M sodium hydroxide at 45°C for two hours to hydrolyse the bonds, then cooled at room temperature, neutralised with 200 µL of concentrated hydrochloric acid and spun at 500 g for ten minutes. The supernatants were adjusted to pH 3.5 with 10 M sodium hydroxide and allowed to equilibrate at 4˚C for 15 minutes, spun at 500 g for five minutes and applied on C18 cartridges (IST, Mid-Glamorgan, UK) which had been preconditioned with 2 ml of ethanol and deionised water. The cartridges were sequentially washed with 2 ml of 15% ethanol and hexane (Merck, San Diego, CA, USA) under slight positive pressure to obtain a flow rate of 0.5 ml/min and finally eluted from the column with 2 ml of ethyl acetate (Scharlau, Barcelona, Spain) and stored at -70˚C.

Levels of 15-F_{2t}-IsoP in the elutes were measured using a competitive immunoassay kit according to the manufacturer's recommendations and previously-published protocol.[7] The elutes were evaporated under a stream of nitrogen and reconstituted in an assay buffer. 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) standards included in the kit were used within 60 minutes of preparation. Elutes and standards were pipetted onto microtitre plates coated with goat anti-rabbit immunoglobulin G. The conjugate (alkaline phosphatase conjugated with 15-F_{2t}-IsoP) and antibody (rabbit polyclonal antibody to 15-F_{2t}-IsoP) were added and incubated at room temperature on a shaker at 500 rpm for two hours. Wells were washed three times with the assay buffer, and the substrate (p-nitrophenyl phosphate) was added and incubated further for 45 minutes without shaking. Finally, the enzyme reaction was stopped by the addition of 50 µL of trisodium phosphate. The intensity of the yellow colour (end-point) is inversely proportional to the concentration of 15-F_{2t}-IsoP in both standards and samples, and the optical density was read at 405 nm using a microplate reader (Bio-Rad 680, Hercules, CA, USA). 15-F_{2t}-IsoP concentrations in the samples were calculated by interpolating the absorbance readings from the standard curve generated with the 15-F_{2t}-IsoP standards. Post hoc Bonferroni test was used to analyse mean values of 15-F_{2t}-IsoP observed in the three different groups, using the Statistical Package for Social Sciences software version 6.0 (SPSS Inc, Chicago IL, USA). $p < 0.001$ was considered statistically significant.

RESULTS

The serum levels of 15- F_{2t}-IsoP were measured in the three groups of individuals, i.e. 25 healthy adults, 25 patients with diabetes mellitus who were otherwise healthy, and 25 patients undergoing treatment for melioidosis in the hospital, as shown in Table I. These results were analysed using the post hoc Bonferroni test (Table II). In the normal healthy controls, the levels ranged from 351.6 to 685.6, with a mean of 510.3 pg/ml In the diabetes mellitus-only group, the range was 937.7 to 1880.8, with a mean of 1357.9 pg/ml, and in the diabetes mellitus cum melioidosis group, the range was 2729.6 to 6856.3, with a mean of 4343.6 pg/ml.

Looking at the individual readings of 15-F_{2t}-IsoP, the highest level of 685.6 pg/ml in the normal group was still lower than the lowest level of 937.7 pg/ml in the diabetic group. Similarly, the lowest reading of 2,729.6 pg/ml in the melioidosis group was higher than the highest reading of 1,880.8 pg/ml in the diabetic group. The lowest mean level of 510.3 pg/ml of 15-F_{2t}-IsoP was detected in the normal healthy group, and the highest,

Table I. Levels of 15-F_{2t}-IsoP.

Patient no.	Serum levels of 15-F_{2t}-IsoP (pg/ml)		
	Normal group	Diabetic group	Melioidosis + DM group
1	611.1	1,023.5	3,134.0
2	381.6	1,253.1	5,835.7
3	413.1	1,115.8	5,201.1
4	525.0	1,407.3	2,729.6
5	570.3	1,491.4	3,855.6
6	480.9	1,675.3	5,971.6
7	416.3	1,448.8	6,856.3
8	685.6	1,052.9	5,446.2
9	625.3	1,148.7	6,110.7
10	583.6	1,083.9	4,853.9
11	351.6	1,827.0	4,227.6
12	413.2	1,992.9	4,853.9
13	701.6	1,535.3	4,037.3
14	485.4	1,182.5	5,971.6
15	370.7	993.7	4,635.5
16	496.7	1,217.3	2,990.8
17	639.9	937.7	3,767.8
18	662.1	1,290.0	3,169.5
19	557.3	965.3	3,457.9
20	463.6	991.0	3,664.3
21	570.3	1,367.1	2,793.1
22	442.7	1,328.0	2,924.8
23	360.2	1,625.9	2,992.9
24	394.6	2,112.1	3,281.6
25	556.0	1,880.8	5,827.6
Mean	510.3	1,357.9	4,343.6

DM: diabetes mellitus

4,343.6 pg/ml, in the diabetic melioidosis patient group. The difference was highly significant ($p < 0.001$). The mean in the melioidosis group was eight times higher than the mean of the normal non-diabetic group. The mean level of 15-F_{2t}-IsoP in the diabetes mellitus-only group was 1,357.9 pg/ml, which is significantly higher than that in the normal group ($p < 0.001$), but significantly lower than that in the melioidosis patients group. In summary, the mean levels of 15-F_{2t}-IsoP in the melioidosis group were significantly higher than in the diabetes mellitus-only group and the normal healthy control group (p-values between the groups were less than 0.001) (Fig. 1).

DISCUSSION

Free radical-mediated oxidation of biological molecules and tissues is associated with a variety of pathological events, such as cancer, ageing and diabetes mellitus. Bacterial infection is another condition that gives rise to excessive free radical production via the respiratory burst following phagocytosis of the invading organisms.[1] Oxidative stress may occur if the overproduction of free

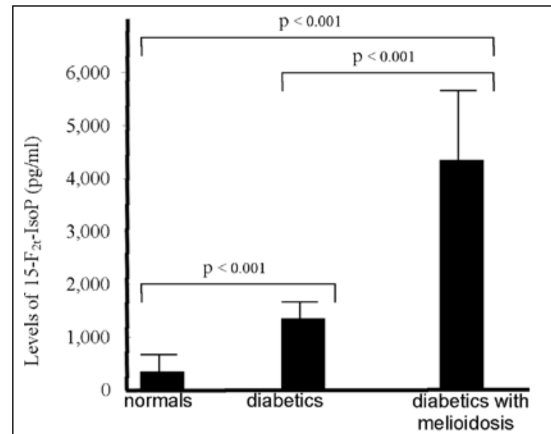

Fig. 1 Bar chart shows the mean serum levels of isoprostane in the three groups.

radicals is not countered by the host antioxidant system, thus leading to impairment of the host cellular functions. IsoPs are not only biomarkers of oxidative stress, but also have biological effects on the respiratory system. This suggests that they might function as pathophysiological mediators of oxidant injury in the lung. Strong positive correlation was observed between 15-F_{2t}-IsoP values obtained by immunoassay and those obtained by gas chromatography/mass spectrometry, but variations among immunoassays can arise due to differences in antibody specificity, extraction protocols and recovery levels. Therefore, comparison of data among laboratories would only be meaningful if a common calibrator is used,[8] hence there are no universally-accepted normal levels of IsoPs for the various biological fluids. The IsoPs levels in the 25 healthy adult blood donors had a range of 351–685 pg/ml and a mean of 510 pg/ml (Table II). This is in accordance with the low levels that are found in normal human biological fluids.

In diabetes mellitus, oxidative stress occurs due to hyperglycaemia and the functional limitation of the hexose monophosphate shunt. This stress alters the plasma lipoprotein profile, and cell membranes undergo lipid peroxidation, which results in enhanced levels of IsoPs. In our diabetic group, the IsoPs levels ranged from 937.7 to 1,880.8 pg/ml, with a mean of 1,357.9 pg/ml.

This was significantly higher than in the normal control group ($p < 0.001$). Davi et al reported similar findings of diabetes mellitus being associated with increased formation of IsoPs as a correlate of impaired glycaemic control and enhanced lipid peroxidation.[9] In patients with diabetes mellitus, an altered oxidative pattern is present not only in the fasting state, but also after food intake,[10] so the timing of obtaining serum samples does not matter for the measurement of IsoPs. As expected, the isoprostane levels were the highest in the melioidosis patients with diabetes mellitus (Fig. 1). All of them were hospitalised and underwent therapy for melioidosis. The mean of 4,343.6 pg/ml was eight-

Table II. Post hoc Bonferroni tests.

Group I	Group J	Mean differences (I-J)	Standard error	95% confidence interval Lower bound	95% confidence interval Upper bound	p-value
Normal	Melioidosis	-3,833.29*	214.26	-4,358.49	-3,308.09	< 0.001
	Diabetic	-847.55*	214.26	-1,372.75	-322.35	< 0.001
Melioidosis	Normal	3,833.29*	214.26	3,308.09	4,358.49	< 0.001
	Diabetic	2,985.74*	214.26	2,460.54	3,510.94	< 0.001
Diabetic	Normal	847.55*	214.26	322.35	1,372.75	< 0.001
	Melioidosis	-2,985.74*	214.26	-3,510.94	-2,460.54	< 0.001

*The mean difference is significant at the 0.05 level

times higher than the control group. The difference was therefore highly significant ($p < 0.001$).

Sepsis is a response to infection. It is characterised by the production of inflammatory cytokines and the release of highly-reactive oxygen and nitrogen intermediates. These oxidising species are thought to contribute to much of the end-stage tissue damage seen in infections.[11] Puthucheary et al had reported that patients with sepsis due to *B. pseudomallei* develop ALI and/or ARDS, which result in high mortality.[6] All the melioidosis patients in the present study had high levels of 15-F_{2t}-IsoP, and although they were all diabetics, the levels were significantly higher than in the diabetes mellitus-only group. Most of them were septicaemic and at risk of developing ALI and/or ARDS. The oxidative stress theory of ARDS proposes that an insult such as severe sepsis, leads to the activation of neutrophils and macrophages, which subsequently release reactive oxygen-free radicals. These radicals may result in lipid peroxidation of endothelial and epithelial cell membrane phospholipids, thereby altering the structure and function of cell membranes.[4] The authors also reported elevated levels of 8-iso-$PGF_{2\alpha}$ in the breath condensate of patients at risk for ALI and/or ARDS, and these levels correlated well with plasma levels of 15-F_{2t}-IsoP. There is now evidence that essentially every cell type in the lung responds in some pathologically relevant way to isoprostanes. As such, they have been elevated from being merely markers of oxidative stress to being pathologically relevant mediators; perhaps they should even be considered as a novel class of inflammatory mediators.[12]

The main finding of the present study was an eight-times higher median total circulating IsoPs levels in patients with diabetes mellitus infected with *B. pseudomallei* when compared with the levels in control subjects. The hierarchy of IsoPs levels was: melioidosis with diabetes mellitus > diabetes mellitus only > healthy controls (Fig. 1). We believe that this is the first report linking *in vivo* oxidative stress status and patients with melioidosis. In our study, we are aware that we have not been able to include patients without diabetes mellitus presenting with melioidosis, and patients with other infectious diseases. In our experience, only children and young adults contract melioidosis without a predisposing condition of diabetes mellitus, and we very rarely see such patients.

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