$iPLA_2$ mRNA Expression by Human Neutrophils in Type 2 Diabetes and Chronic Periodontitis

Srinivas Ayilavarapu[1], Alpdogan Kantarci[2], Hatice Hasturk[2] and Thomas E. Van Dyke[2]

[1]Department of General Dentistry, Boston University Henry M. Goldman School of Dental Medicine, Boston, MA 02118. [2]Department of Applied Oral Sciences, Forsyth Institute, Cambridge, MA 02142

Abstract

Type 2 diabetes mellitus (T2D) is becoming increasingly prevalent worldwide and complications of T2D cause significant systemic and dental morbidity in the susceptible individual. Although T2D has been linked as a significant risk factor for chronic periodontitis (CP), molecular mechanisms explaining the pathogenesis and inflammatory impact of CP in T2D are lacking. $iPLA_2$ is the calcium-independent form of phospholipase A_2. In previous studies, we demonstrated that $iPLA_2$ enzyme activity is altered in T2D. The purpose of this study was to elucidate the level of the $iPLA_2$ abnormality in T2D by measuring messenger RNA levels in T2D-associated CP. A total of 53 healthy and T2D subjects with CP were recruited for this study. The clinical periodontal exam included probing pocket depth, clinical attachment levels and bleeding on probing. Peripheral venous blood was collected and neutrophils were isolated. Real time polymerase chain reaction was used to quantify $iPLA_2$ mRNA in neutrophils from healthy controls and people with diabetes. Results revealed that the prevalence of moderate to severe CP was increased in people with T2D. The $iPLA_2$ mRNA levels in diabetics with different severity of CP were not significantly different compared to healthy controls; 1.07 vs 0.97 (mild CP), 1.07 vs 0.85 (moderate CP) and 1.07 vs 1.05 (severe CP). Collectively, the data suggest that levels of $iPLA_2$ mRNA in T2D are not different than in health and are not directly influenced by periodontal disease status. The impact of inflammation on $iPLA_2$ regulation is at the level of activation of the enzyme rather than expression at the mRNA level.

Key words: Diabetes, inflammation, chronic periodontitis, neutrophils, phospholipase A_2

Introduction

Chronic periodontitis (CP) is an inflammatory condition of the supporting structures of the teeth that is initiated by bacteria, but the host response determines the outcome. Chronic periodontitis is the result of a failure of resolution of the inflammation and is thought to exacerbate systemic inflammation in conditions such as T2D, atherosclerotic heart disease, premature birth in pregnant women with periodontitis, and osteoarthritis (Fredman *et al.*, 2011). Current epidemiology suggests that 47% of the United States population has CP (Eke *et al.*, 2012) and therefore it is considered to be a major public health concern.

Data from the 2011 National Diabetes Fact Sheet reports that about 25.8 million (8.3% of the population) have T2D and about 79 million have pre-diabetes (Center for Disease Control and Prevention, 2011). The total economic burden of T2D is about $178 billion (Center for Disease Control and Prevention, 2011). Among young adults, those with T2D have about twice the risk of CP compared to those without T2D (Center for Disease Control and Prevention, 2011). Adults aged 45 years or older with poorly controlled T2D [glycated hemoglobin A1c (HbA1c) > 9%] are 2.9 times more likely to have severe CP than those without T2D. About one-third of people with T2D have severe forms of CP consisting of loss of attachment (≥ 5 mm) (Center for Disease Control and Prevention, 2011).

The biologic factors linking inflammatory pathology in CP and T2D are poorly understood. Microvascular injury at the affected target organ/tissue due to AGE (advanced glycation end products) - RAGE (receptor for advanced glycation end products) interactions at the cellular level (Ding *et al.,* 2007b) has been implicated as a major mediator of diabetic complications. In macro-

Correspondence to: Thomas E. Van Dyke, DDS, PhD. Department of Applied Oral Sciences, Forsyth Institute, Cambridge, MA, USA, 13018. E-mail: tvandyke@forsyth.org

phages, AGE-RAGE interactions result in increased pro-inflammatory cytokine secretion and superoxide generation (Ding *et al.,* 2007a; Ding *et al.,* 2007b). Priming of neutrophils in T2D contributes to increased reactive oxygen species (ROS) activity and oxidative stress, causing microvascular damage via the RAGE pathway involving protein kinase C (PKC) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Karima *et al.*, 2005). More specifically, increased, activity and a premature assembly of NADPH oxidase components, particularly $p47^{phox}$, in the cytoplasmic membrane in the primed neutrophil causes a more robust superoxide anion generation that results in tissue injury (Omori *et al.*, 2008). Studies have also shown that neutrophils from diabetic mice are primed for an exaggerated inflammatory response and impaired transmigration (Gyurko *et al.*, 2006). Recently, the role of $iPLA_2$ as a regulatory enzyme in the excessive generation of superoxide from neutrophils of diabetic subjects was identified (Ayilavarapu *et al.*, 2010). In this study, there was increased activity of iPLA2 in neutrophils from poorly controlled diabetics. The iPLA2 pathway was implicated, in part, in the increased oxidative burst, which was likely due to increases in secondary messenger levels of arachidonic acid (AA). Arachidonic acid has been previously shown to be associated with the activation of preassembled NADPH oxidase (Dana *et al.*, 1994). It is also further metabolized by specific oxygenases, leading to the generation of pro-inflammatory lipid mediators such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4). Increased levels of both PGE_2 and LTB_4 have been associated with bone loss of periodontitis (Hasturk *et al.*, 2007). The increased inflammatory burden induced by the presence of periodontal disease in people with type 2 diabetes may pose significant risk for complications of T2D, such as cardiovascular disease. Identification of regulatory pathways and signaling molecules that connect these two inflammatory diseases will potentially provide better understanding and more efficient therapeutic approaches. In this study, we further characterize the relationship between altered $iPLA_2$ enzyme activity in T2D and T2D-associated CP. Our findings suggest that $iPLA_2$ enzyme activity co-segregates with T2D-associated CP in the T2D population, but $iPLA_2$ mRNA levels in neutrophils in people with T2D-associated CP do not differ.

Materials and methods

Reagents

N-formyl-methionyl-leucyl-phenylalanine (fMLP), S100B (S100 calcium binding protein B - a RAGE ligand), cytochrome C and propranolol hydrochloride were purchased from Sigma (St. Louis, MO, USA). A23187, pyrrolidine-1 and BEL were purchased from EMD-Biosciences (San Diego, CA, USA). Rabbit polyclonal antiserum to iPLA2 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Trizol reagent was purchased from Invitrogen Corporation (Carlsbad, CA, USA). High capacity cDNA reverse transcriptase kits, real time PCR probes for β-actin, iPLA2 and master mix were obtained from Applied Biosystems (Foster City, CA, USA).

Subject recruitment

A total of 53 subjects including healthy people (n = 27) and people with T2D (n = 26) were recruited for the study. The inclusion and exclusion criteria for T2D, healthy controls and periodontal examination were previously described (Karima *et al.*, 2005). Healthy subjects were recruited from a healthy cohort without T2D and CP. Demographic characteristics, including periodontal and diabetic status, were identified. All study participants received a standard clinical oral examination, including dental and periodontal exam and periapical and bite wing radiographs, to determine their periodontal status. Subjects with T2D were recruited from collaborating community health care or hospital clinics (Dorchester House Multi-Service Center, Dorchester, MA, Codman Square Health Center, Dorchester, MA, Manet Community Health Center, Quincy, MA and Boston University Family Medicine and Endocrinology, Diabetes and Nutrition clinics, Boston, MA). Age-, gender- and ethnicity-matched healthy controls were recruited from the Boston University Goldman School of Dental Medicine community. All subjects signed a Boston University Medical Center Institutional Review Board (BUMC IRB # H-24481)-approved informed consent prior to clinical exam and any other study procedures.

Both female and male subjects at least 18 years old, diagnosed with T2D at least 6 months prior to enrollment and in diabetic maintenance were recruited for the study. Subjects with acute infectious diseases (e.g., tuberculosis), immunocompromised diseases (e.g., AIDS) or receiving chemotherapy were excluded. Chronic use of antibiotics, steroids and non-steroidal anti-inflammatory drugs were also exclusion criteria. A clinical periodontal exam, including probing pocket depth, clinical attachment levels and bleeding on probing was performed on all subjects. Routine full mouth radiographs were obtained to support the clinical diagnosis of CP. Type 2 diabetes subjects were classified as well and poorly controlled based on their HbA1c levels (ADA, 2004). Periodontal status was classified as healthy, gingivitis, mild, moderate or severe CP based on periodontal attachment loss (Armitage, 1999).

Neutrophil isolation

Neutrophils were isolated as described previously (Ayilavarapu *et al.*, 2010). Briefly, peripheral venous blood was collected into heparinized tubes and neutrophils

were isolated by Ficoll-Hypaque gradient centrifugation. Contaminating red blood cells were hypotonically lysed; cell preparations were routinely 99% neutrophils with ≥ 95% viability, as determined by trypan blue exclusion.

Quantitative Real Time PCR (qPCR)

Total RNA was extracted from neutrophils using Trizol reagent (Invitrogen) per manufacturer's protocol. The concentration and purity of RNA was estimated by the A_{260}/A_{280} ratio spectrophotometrically (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA). The A_{260}/A_{280} ratio was routinely above 1.6. One microgram (μg) RNA was converted to cDNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems). The reaction mixtures were thoroughly mixed and kept at 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 0.05 minutes in a thermal cycler (ABI 9700, Applied Biosystems, Foster City, CA, USA). qPCR was performed using primers and TaqMan probes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and iPLA2 (Group VI) labeled with FAM (fluorescein amidite) dye (Applied Biosystems, TaqMan gene expression assays). Beta-actin was selected as an internal control and amplified using pre-formulated VIC-TAMRA (5-carboxy tetramethylrhodamine) labeled TaqMan probes (Applied Biosystems, Endogenous Control). Quantification was performed in an automated thermal cycler (ABI Prism 7000 Sequence Detector, Applied Biosystems, Foster City, CA, USA). The reaction mixtures were kept at 50ºC for 2 minutes (1 cycle), 95ºC for 10 minutes (1 cycle), and 95ºC for 15 seconds (45 cycles) and finally 60ºC for 1 minute. The results were analyzed through a software interface and spreadsheet for the calculation of relative expression (GAPDH/β-actin or iPLA2/β-actin). Relative changes of iPLA2 mRNA levels were analyzed using the 2^-ΔΔCt method.

Statistical analysis

All data are presented as the average of at least three experiments with standard error of the mean (SEM) unless otherwise stated. For comparison between two groups, Student's *t*-test was applied for significance, and for multiple group analysis one-way ANOVA (analysis of variance) was used with post-hoc Bonferroni or LSD (least significance differences) corrections.

Results

Severity of CP is associated with diabetes

The demographics of this study population are presented in *Table 1*. The subject population consisted of 26 T2D and 27 healthy controls. Among the subjects with diabetes, the number with CP (18) was significantly higher than those with milder disease (8; $p < 0.05$). Of the 18 with CP, 13 were moderate to severe.

Expression of iPLA$_2$ mRNA in neutrophils from patients with T2D and CP

iPLA$_2$ mRNA levels were not significantly different in neutrophils taken from people with both T2D and CP compared to healthy controls (*Figure 1*). There was a trend to higher iPLA$_2$ mRNA expression with worsening of periodontal disease in T2D. However, none was different from control.

Table 1. Subject demographics: Among the subjects with diabetes, the number with chronic periodontitis (18) was significantly higher than those with milder disease (8).

		Healthy	Diabetes
n		27	26
Mean age (years)		36.5 ± 9.41	53.22 ± 10.1
Gender	Male	17	17
	Female	10	9
Ethnicity	Caucasian	14	12
	African-American	8	12
	Asian	3	
	Hispanic	2	2
Mean HbA1c (%)			8.2
HbA1c range (%)			5.9-12.7
Periodontal status	Healthy/gingivitis		8*
	Mild CP		5
	Moderate/severe CP		13

*$p < 0.05$. CP, chronic periodontitis; HbA1c, glycated hemoglobin

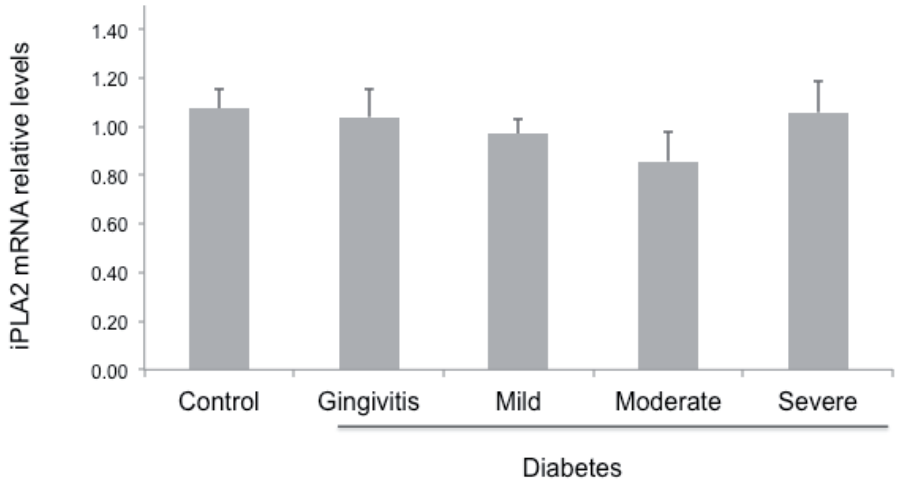

Figure 1. iPLA2 mRNA levels in neutrophils of people with type 2 diabetes (T2D) and chronic periodontitis [CP; grouped as gingivitis (n = 8), mild CP (n = 5), moderate CP (n = 5) and severe CP (n = 8)] compared to healthy controls (n = 27). The levels of iPLA2 mRNA were compared in healthy controls to each periodontal status in diabetics by analysis of variance (ANOVA). Relative levels of iPLA2 mRNA are presented as mean ± standard error of mean.

Discussion

A large number of studies have shown a strong association between T2D and CP (Tsai *et al.*, 2002; Taylor and Borgnakke, 2008). Altered neutrophil function has been implicated as a mediator of the complications of T2D via the production of excessive superoxide (Gyurko *et al.*, 2006; Ayilavarapu *et al.*, 2010) causing oxidative stress and tissue damage. Increased PKC activity in diabetic neutrophils enhances superoxide release, which correlates with worsening glycemic control in these individuals (Karima *et al.*, 2005). There is also a significant correlation between blood glucose levels and severity of CP in T2D, suggesting that increased oxidative stress and increased inflammation are involved in both of the diseases. The combined presence of T2D and CP increases the risk for subclinical atherosclerotic heart disease and coronary heart disease (Southerland *et al.*, 2012).

The data presented in this study confirm previous observations of an association between T2D and CP. This study was designed to further characterize the role of neutrophil $iPLA_2$ in T2D-associated CP. The aim of the study was to determine the subcellular level of the increased risk of elevated $iPLA_2$ in T2D and CP by determining regulation of mRNA. $iPLA_2$ has been shown to be a key mediator of acute inflammation in animal models (Gilroy *et al.*, 2004). In humans, $iPLA_2$ regulates several conditions such as diabetes, cancer and inflammation, and has been suggested as a novel and important target for drug development (Wilkins and Barbour, 2008). $iPLA_2$ has also been identified as a novel regulatory molecule that promotes inflammation in brain (Strokin *et al.*, 2011) and in acute pulmonary inflammation (Rastogi and McHowat, 2009). Recently, we showed increased activity at the enzyme level of $iPLA_2$ in neutrophils from people with T2D (Ayilavarapu *et al.*, 2010). Thus, $iPLA_2$ has been determined to be a key mediator of inflammation.

Various studies have suggested that CP, as an infection-initiated inflammation, affects glycemic control in people with T2D (Engebretson *et al.*, 2013). However, mechanisms by which CP can affect glycemic control are not well understood. Increased oxidative stress due to CP might be one possible explanation (Taylor *et al.*, 2013). Therefore, a complete understanding of the $iPLA_2$ pathway assumes great significance. In this study, we explored the role of $iPLA_2$ mRNA expression levels as a marker of the neutrophil priming mechanism in people with T2D and CP.

Data presented in *Figure 1* show that neutrophils from people with T2D-associated CP exhibited no significant change in the expression of iPLA2 mRNA compared to healthy controls and that it was independent of severity of disease. The data suggest that the increased activity of $iPLA_2$ enzyme is due to post-translational activation of the enzyme in neutrophils locally, as $iPLA_2$ levels at the mRNA level do not differ in health and disease and are independent of enzyme activation. The role of different isoforms of $iPLA_2$ in the neutrophil oxidative burst remains to be explored.

In *Figure 2*, we present a working model for neutrophil priming via $iPLA_2$ activation in people with T2D. Briefly, when a neutrophil is challenged by a bacterial

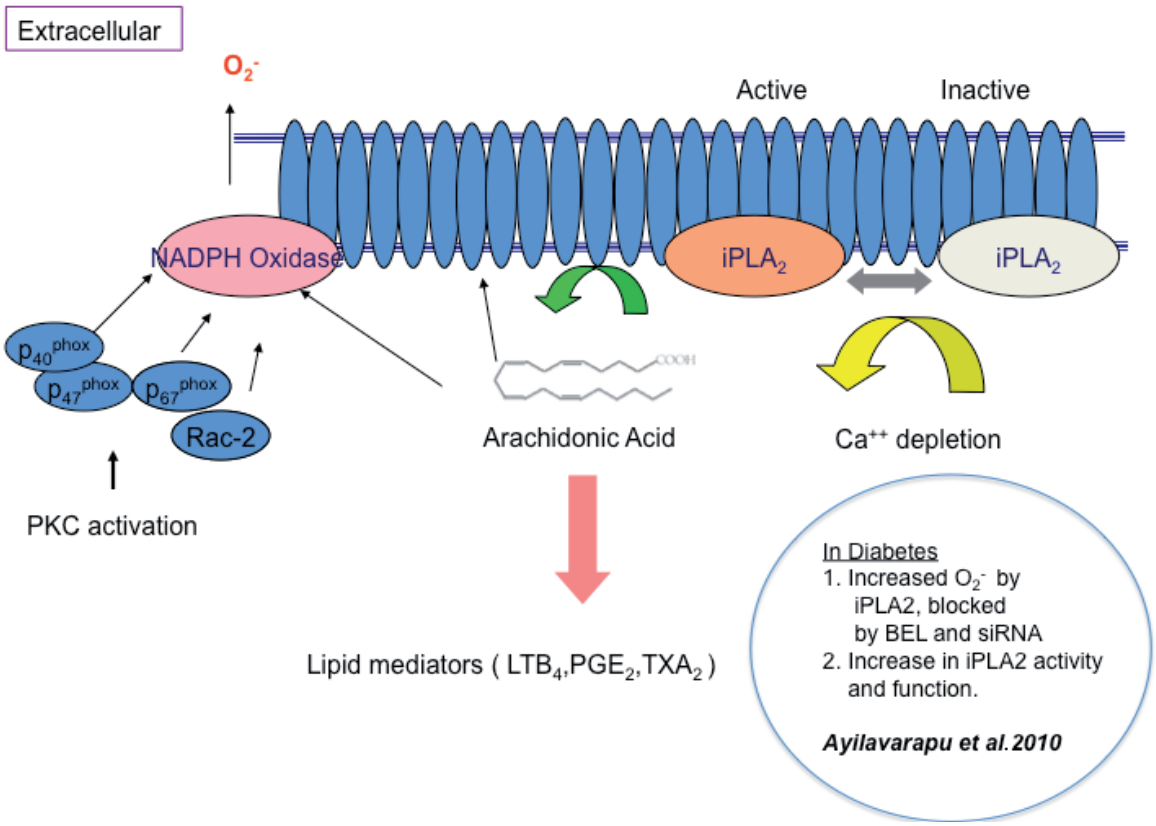

Figure 2. Suggested model of the pathway for increased oxidative stress in neutrophils from people with type 2 diabetes (T2D). A cross-section of a neutrophil membrane is represented in the presence of hyperglycemia. Receptor for advanced glycation endproducts (RAGE) binding of AGE and high glucose increase the expression of iPLA$_2$, premature translocation of p47^{phox} and activation NADPH-oxidase with phosphorylation of protein kinase C (PKC) upon stimulation with fMLP ligand. Increased iPLA$_2$ activation results in increased free arachidonic acid, which is an important messenger for superoxide formation and oxidative stress. The inflammation due to increased oxidative stress in people with T2D is an important risk factor for chronic periodontitis.

ligand such as fMLP, a signaling cascade is initiated via PKC phosphorylation and activation of NADPH oxidase and iPLA$_2$. This signaling mechanism results in superoxide generation by the neutrophil. In people with T2D, the NADPH-oxidase is pre-assembled and increased levels and activation of PKC and iPLA$_2$ result. Taken together, the data from this study and from our previous work (Ayilavarapu *et al.*, 2010) suggest that increased enzyme activity of iPLA$_2$ is a post-translational event and that iPLA$_2$ mRNA levels are tightly controlled in both health and disease.

Larger studies are required to determine the generalizability of these observations. It will also be necessary to determine iPLA$_2$ mRNA levels in CP subjects without T2D, although the lack of differences between groups in this study suggest that these will be similar. Future studies will explore the impact of CP on compounding increased systemic inflammation in T2D.

Acknowledgment

This work was supported in part by USPHS grants DE-15566, DE-16933, DE-18917, and RR-00533.

References

American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2004; **27:**S5-S10.

Armitage GC. Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* 1999; **4**:1-6.

Ayilavarapu S, Kantarci A, Fredman G, *et al.* Diabetes-induced oxidative stress is mediated by Ca^{2+}-independent phospholipase A$_2$ in neutrophils. *Journal of Immunology* 2010; **184**:1507-1515.

Center for Disease Control and Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States. U.S. Department of Health and Human Services, 2011. http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2011.pdf

Dana R, Malech HL, and Levy R. The requirement for phospholipase A_2 for activation of the assembled NADPH oxidase in human neutrophils. *Biochemistry Journal* 1994; **297(Pt 1)**:217-223.

Ding Y, Kantarci A, Badwey JA, Hasturk H, Malabanan A and Van Dyke TE. Phosphorylation of pleckstrin increases proinflammatory cytokine secretion by mononuclear phagocytes in diabetes mellitus. *Journal of Immunology* 2007a; **179**:647-654.

Ding Y, Kantarci A, Hasturk H, Trackman PC, Malabanan A and Van Dyke TE. Activation of RAGE induces elevated O_2- generation by mononuclear phagocytes in diabetes. *Journal of Leukocyte Biology* 2007b; **81**:520-527.

Eke PI, Dye BA, Wei L, *et al.* Prevalence of periodontitis in adults in the United States: 2009 and 2010. *Journal of Dental Research* 2012; **91**:914-920.

Engebretson S, Gelato M, Hyman L, Michalowicz BS and Schoenfeld E. Design features of the Diabetes and Periodontal Therapy Trial (DPTT): a multicenter randomized single-masked clinical trial testing the effect of nonsurgical periodontal therapy on glycosylated hemoglobin (HbA1c) levels in subjects with type 2 diabetes and chronic periodontitis. *Contemporary Clinical Trials* 2013; **36**:515-526.

Fredman G, Oh SO, Ayilavarapu S, Hasturk H, Serhan CN and Van Dyke TE. Impaired phagocytosis in localized aggressive periodontitis: rescue by resolvin E1. *PLoS One* 2011; **6**:e24422.

Gilroy DW, Newson J, Sawmynaden P, Willoughby DA and Croxtall JD. A novel role for phospholipase A_2 isoforms in the checkpoint control of acute inflammation. *The FASEB Journal* 2004; **18**:489-498.

Gyurko R, Siqueira CC, Caldon N, Gao L, Kantarci A and Van Dyke TE. Chronic hyperglycemia predisposes to exaggerated inflammatory response and leukocyte dysfunction in Akita mice. *The Journal of Immunology* 2006; **177**:7250-7256.

Hasturk H, Kantarci A, Goguet- Surmenian E, *et al.* Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis *in vivo*. *The Journal of Immunology* 2007; **179**:7021-7029.

Karima M, Kantarci A, Ohira T, *et al.* Enhanced superoxide release and elevated protein kinase C activity in neutrophils from diabetic patients: association with periodontitis. *Journal of Leukocyte Biology* 2005; **78**:862-870.

Omori K, Ohira T, Uchida Y, *et al.* Priming of neutrophil oxidative burst in diabetes requires preassembly of the NADPH oxidase. *Journal of Leukocyte Biology* 2008; **84**:292-301.

Rastogi P and McHowat J. Inhibition of calcium-independent phospholipase A_2 prevents inflammatory mediator production in pulmonary microvascular endothelium. *Respiratory Physiology and Neurobiology* 2009; **165**:167-174.

Southerland J, Moss HK, Taylor G, *et al.* Periodontitis and diabetes associations with measures of atherosclerosis and CHD. *Atherosclerosis* 2012; **222**:196-201.

Strokin M, Sergeeva M and Reiser G. Proinflammatory treatment of astrocytes with lipopolysaccharide results in augmented Ca^{2+} signaling through increased expression of via phospholipase A_2 (iPLA$_2$). *American Journal of Physiology. Cell Physiology* 2011; **300**:C542-549.

Taylor GW and Borgnakke WS. Periodontal disease: associations with diabetes, glycemic control and complications. *Oral Diseases* 2008; **14**:191-203.

Taylor JJ, Preshaw PM and Lalla E. A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. *Journal of Periodontology* 2013; **84(4 Suppl)**:S113-134.

Tsai C, Hayes C and Taylor G. Glycemic control of type 2 diabetes and severe periodontal disease in the US adult population. *Community Dentistry and Oral Epidemiology* 2002; **30**:182-192.

Wilkins WP 3rd and Barbour SE. Group VI phospholipases A_2: homeostatic phospholipases with significant potential as targets for novel therapeutics. *Current Drug Targets* 2008; **9**:683-697.