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Cyclooxygenase 2, toll-like receptor 4 and interleukin 1β mRNA expression in atherosclerotic plaques of type 2 diabetic patients

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Abstract

Objectives and design   Inflammation has a prominent role in the development of atherosclerosis. Type 2 diabetes could contribute to atherosclerosis development by promoting inflammation. This status might accelerate changes in intrinsic vascular wall cells and favor plaque formation. Cyclooxygenase 2 (COX-2) is highly expressed in atherosclerotic plaques. COX-2 gene expression is promoted through activation of toll-like receptor 4 (TLR4) and pro-inflammatory cytokine interleukin 1β (IL1-β). Aim of this study is to investigate whether expression profiles of pro-inflammatory genes such as COX-2, TLR4 and IL1-β in atherosclerotic plaques are altered in type 2 diabetes (T2D).

Methods   Total RNA was isolated from plaques of atherosclerotic patients and expression of COX-2, TLR4, IL1-β analyzed using real-time PCR. Histological analysis was performed on sections of the plaque to establish the degree of instability.

Results   Statistically significant differences in mRNA expression of COX-2 and IL1-β were found in plaques of T2D compared with non-T2D patients. A multi-variable linear regression model suggests that COX-2 mRNA expression is affected by T2D pathology and IL1-β mRNA expression in atherosclerotic plaques.

Conclusions   Our results support the hypothesis that T2D pathology contributes in vivo to increase the inflammatory process associated with the atherosclerotic plaque formation, as shown by an increment of COX-2 and IL1-β mRNA expression.

Keywords   Cyclooxygenase 2 · Inflammation · Type 2 diabetes · Atherosclerosis

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Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall [1] that affects 346 million people worldwide and a cause of morbidity for 70 % of patients with type 2 diabetes (T2D) [2].

T2D accelerates the clinical course of atherosclerosis [3] damaging mesangial and endothelial cells [4]. Arterial endothelial cells normally resist attachment of the white blood cells streaming past them. However, when subjected to irritative stimuli, arterial endothelial cells express chemoattractant molecules that beckon leukocytes to attach and

migrate through the endothelial monolayer and enter the arterial intima [5]. Irritative stimuli act as pro-inflammatory agents that alter the behavior of intrinsic vascular wall cells and trigger the production of prostanoids, a family of pleiotropic lipid mediators, involved in vascular inflammation [6]. Important irritative stimuli that play a role in atherosclerosis are dyslipidemia, [7, 8] hypertension, [9, 10] cigarette smoke [11, 12] and diabetes (type 1 and 2) [3, 13, 14]. Cyclooxygenase 2 (COX-2) is the enzyme responsible for the high output production of prostanoids (prostaglandins and thromboxanes) from arachidonic acid in multiple inflammatory pathophysiological conditions [15]. In particular, prostaglandin E2 (PGE2) plasma levels are increased in inflammatory processes, like atherosclerosis [16]. COX-2, an inducible enzyme that is normally absent from tissues (with the exception of kidney and parts of the brain, where COX-2 is constitutive) [17], is highly expressed in atherosclerosis lesions [18] and its expression is induced by a wide range of inflammatory stimuli. Inflammation has a prominent role in the development of atherosclerosis, and PGE2 expression may provide a link between risk factors, such as T2D [19], and the cellular alterations that underlie the disease [5] and vascular complications [20]. In particular, Sheu et al. [21] and Kellogg et al. [22] observed that high glucose concentration can up-regulate COX-2 expression with the induction of PGE2 in myocardial cells and umbilical vein endothelial cells. Several studies suggest that COX-2 expression is promoted through activation of toll-like receptor 4 (TLR4) [23, 24]. Recently, Ferronato et al. [25] reported a coordinated up-regulation of COX-2 and TLR4 expression in peripheral blood of stroke patients, suggesting that TLR4 might participate in the peripheral inflammatory process after stroke in part through a COX-2 dependent pathway. It has been reported that high glucose concentration promotes the expression of COX-2 through the activation of IL1-β in mesangial cells [26] and islets of Langerhans [27]. Although there is mounting evidence that T2D contributes to the pathophysiology of atherosclerosis, knowledge of the mechanism of action in the inflammatory pathway is lacking. The aim of this study is to investigate whether the mRNA expression profile of the pro-inflammatory genes COX-2, TLR4 and IL1-β is altered in T2D patients and whether this altered expression could be involved in the augmented inflammatory process in the plaques of these patients.

Subjects and methods

Patients

Consecutive eligible patients with internal carotid artery stenosis undergoing carotid endarterectomy at Verona University Hospital were included in this study. A total of 60 patients, as detailed in Table 1, were selected for gene expression analysis. They were all of Caucasian descent from North-East Italy. The group included 23 patients who presented transient ischemia attack (TIA) or ischemic stroke and 37 with asymptomatic carotid plaques. In the group of TIA and ischemic stroke six subjects had T2D. In the asymptomatic carotid plaque group, eight subjects had T2D (Table 1). The atherothrombotic nature of the event was established as described by Ferronato et al. [25].

Characteristics of the patients, vascular risk factors, current therapy information, and plaque histological analysis, are given in Table 2. Exclusion criteria included intracerebral hemorrhage, inflammatory pathologies, and non-steroidal or glucocorticoid anti-inflammatory therapy. Smoking habit was defined as non or current smoker. Current smokers were defined as individuals who smoked any tobacco in the past 12 months and included those who had quit within the past year. Non-smokers were defined as subjects who never smoked. Ex-smokers were defined as those who had quit more than a year earlier [28] and, if they had quit more than 10 years earlier, they were considered non-smokers [29]. The study was approved by the Ethical Committee of the Hospital and informed written consent was obtained from all patients before enrollment.

RNA extraction from carotid plaques

Carotid plaques were retrieved from patients during carotid endarterectomy and immediately placed in RNA later solution (Ambion Inc) for RNA stabilization and protection. After being placed in RNA later and kept in ice, the sample was taken to the laboratory by a staff member to be processed. Total RNA from carotid plaques was purified by ultracentrifugation in cesium chloride gradient.

Quantitative real-time PCR

Total RNA from whole carotid plaques was extracted by a guanidinium thiocyanate/cesium chloride gradient method and RNA from samples was reverse transcribed using a complementary DNA (cDNA) synthesis kit (Invitrogen), according to the supplier's instructions. TLR4, COX-2 and IL1-β gene expressions were determined by real-time RT-PCR using Sybr Green I as described by Ferronato et al. [25]. PCR were performed in triplicate for each gene. Quality control of the RNA was tested using non-denaturing agarose gel electrophoresis verifying the integrity of the 18S RNA.

Histological analysis

Surgically removed carotid plaques were frozen in isopentane and preserved at −80 °C for histopathological analysis.

Serial sections of the plaque were analyzed with hematoxylin and eosin to evaluate fibrous cap integrity, intraplaque hemorrhage, calcium deposits, inflammatory infiltrate, neovascularization lipid core and endoluminal thrombus. For immunohistochemistry, obtained cryostat sections were fixed in acetone at −20 °C for 10 min, pre-incubated in PBS with 3 % hydrogen peroxide and, after three washes, were incubated in PBS with monoclonal mouse anti-human CD-68 (Oncogene), anti-human HLA II (Dako) and anti-human CD3 (Thermo Scientific). As secondary antibodies, biotinylated antibodies IgG anti-rabbit and anti-mouse (Dako Corporation, Carpinteria, CA) were used. After washing in PBS, the sections were treated with ABC-Elite (Vector) and the peroxidase activity highlighted by development in peroxidase substrate kit (Vector). Degree of plaque instability was established on the basis of inflammatory infiltrate, according to Lovett et al. [30]. For each plaque, the presence and/or amount of the following features was recorded on a simple grade scale: rupture of the fibrous cap, lipid core size, nodular calcification, neovascularization, inflammatory infiltrate, infiltration of the fibrous cap, proportion of fibrous tissue, intraplaque hemorrhage, surface thrombus, number of foam cells and overall instability.

Statistical analysis

Statistical analysis was managed with R version 2.13.1 (www.r-project.org). The Shapiro−Wilk test was applied to check for normal distribution and the Bartlett test to check for equal variances across samples. When not normally distributed, values were logarithmically transformed. Differences in gene expression were analyzed with Student's t test and the values were given as mean ± standard deviation (SD). A multiple linear regression was fitted to the data to determine the significance of covariates through the stepwise method of R package 'MASS' version 7.3–19. Hypertension and dyslipidemia were adjusted for the patient's therapies by adding the covariates (statins and ACE inhibitor) to the regression model for each gene expression level. The likelihood ratio test was performed using the R package 'epicalc' version 2.15.1.0. The Baron and Kenny [31] analytical procedure was used to determine the mediator function of a third variable. The R package 'pwr' version 1.1.1 was used to check the statistical power based on the sample size, standard deviation and mean to have type 2 error (beta) >80 %. A p-value < 0.05 (type 1 error or alpha <0.05) was considered statistically significant.

Results

In this study, we analyzed gene expression of COX-2, TLR4, and IL1-β in atherosclerotic plaques in a group of 60 atherosclerotic patients. Sixteen were T2D and 43 were non-T2D patients (missing data for one patient) (Table 1). Along with T2D, other risk factors as smoking habit (mean of cigarette exposure in current smokers is 29 ± 19 cigarettes/day). All the ex-smokers quit more than 10 years earlier and then considered as non-smokers, hypertension, dyslipidemia, sex and age were considered in the analysis. No differential mRNA expression was observed between symptomatic and asymptomatic patients for all the three genes analyzed (p-value > 0.05, data not shown).

In addition, considering each degree of plaque instability (grade 1 = stable, grade 2 = probably stable, grade 3 = probably unstable), no differential COX-2, TLR4, and IL1-β mRNA expression was observed when comparing asymptomatic with symptomatic patients. Thus, symptomatic and asymptomatic patients were considered as one group.

COX-2 and IL1-β mRNA expressions were higher in carotid plaques of patients with T2D compared to non-T2D patients (1.67 ± 1.45 vs. 0.84 ± 0.72, $p < 0.01$; 3.25 ± 2.41 vs. 1.04 ± 0.77, $p < 0.01$, respectively). Smoking habit increased significantly the expression of COX-2 (1.57 ± 1.27 vs. 0.68 ± 0.53, $p < 0.01$). TLR4 mRNA expression did not show statistical difference in any of the risk factors considered (Table 3).

Correlation analysis in relation to T2D status

In T2D patients, a linear correlation between COX-2 and TLR4 mRNA expression in plaques was observed ($R = 0.71$, $p < 0.001$), which was not observed in non-T2D patients ($R = 0.18$, $p > 0.05$). No correlation of COX-2 and TLR4 mRNA expression was observed for any of the other non genetic risk factors analyzed (data not shown). A correlation was observed between COX-2 and IL1-β mRNA expression in T2D ($R = 0.69$, $p < 0.05$) and non-T2D patients ($R = 0.49$, $p < 0.001$). Although the linear correlation in non-T2D patients appeared weaker, the difference between the two groups was not statistically significant as determined by the Pearson correlation.

mRNA expression and plaque instability

COX-2 and IL1-β

In the entire population, considering the degree of the overall plaque instability, a statistically significant linear correlation between COX-2 and IL1-β mRNA expression was observed in grade 2 ($R = 0.61$, $p = 0.001$) and grade 3 ($R = 0.75$, $p = 0.03$). Considering foam cells composition of the plaques (calculated as the number of cells: grade 1 = none, grade 2 = <50, grade 3 = at least 50), a linear correlation in mRNA expression was observed in grade 1

Table 1 Characteristics of the study patients

	T2D[a] (n = 16)		non-T2D (n = 43)	
	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
Female	2	4	1	10
Male	5	5	17	15
Age (mean ± SD)	70 ± 10	73 ± 7	70 ± 11	73 ± 7
	72 ± 9		72 ± 9	
Risk factors				
Smoking habit (29 ± 19 cigarettes/day)	4/7	3/9	8/18	7/25
Hypertension	6/7	8/9	11/18	22/25
On ace inhibitor therapy	4/6	7/8	8/11	8/22
Dyslipidemia	5/7	6/9	9/18	19/25
On statin therapy	3/5	3/6	8/9	10/19

Clinical information, risk factors and current therapy divided in T2D and non-T2D patients. Symptomatic patients presented transient ischemia attack (TIA) or ischemic stroke. Asymptomatic patients did not present TIA or ischemic stroke at the time they underwent carotid endarterectomy

ACE angiotensin converting enzyme, *n* number of subjects, *T2D* type 2 diabetes, *Yrs* years

[a] Missing information for 1 patient

Table 2 Characteristics of the study patients

	Grade			
	1	2	3	4
	n T2D subjects/*N* subjects			
Plaque instability	4/14	7/34	4/9	0
Foam cells	6/28	3/12	4/9	0
Lipid core	3/7	5/19	6/19	0
Hemorrhage	8/10	2/12	4/18	0
Surface thrombus	9/25	1/4	4/11	0
Fibrous tissue	1/8	10/25	3/7	0
Neovascularization	10/26	2/10	2/4	0
Calcification	0/2	3/11	11/27	0
Rupture fibrous cap	2/8	4/12	8/20	0
Inflammatory cells	1/4	4/17	7/17	1/4

Histological analysis. Number of patients with T2D divided into the grades of histological features. Characteristics of the degree of plaque instability were established on the basis of inflammatory infiltrate, according to Lovett et al. [19]

n subjects = total number of subjects for each grade

($R = 0.50$, $p = 0.04$) and grade 3 ($R = 0.99$, $P = 0.003$) (Table 4). No significant correlation was found for the other histological features ($p > 0.05$, data not shown).

COX-2 and TLR4

In the entire population, an increased linear correlation was observed between COX-2 and TLR4 mRNA expression moving from grade 2 ($R = 0.43$, $p = 0.009$) to grade 3 ($R = 0.69$, $p = 0.03$) considering the degree of the overall plaque instability. Considering the degree of foam cells, we observed a statistically significant linear correlation only in grade 1 ($R = 0.51$, $p = 0.01$) (Table 4). No significant correlation was found for the other histological features ($p > 0.05$, data not shown).

mRNA expression quantification

When stratifying by the degree of the plaque instability in grade 3 plaque instability group, a significant overexpression of COX-2, TLR4 and IL1-β was observed in T2D patients compared to non-T2D patients. When stratifying by the degree of foam cells, we observed a higher COX-2 and IL1-β mRNA expression level ($p < 0.01$ for both) in T2D compared to non-T2D patients [Fig. 1]. No statistically different mRNA expression was observed for any of the other histological features and risk factors analyzed (data not shown).

Linear regression analysis

In the stepwise analysis, the presence of TLR4 and IL1-β mRNA expression along with T2D status and smoking habit showed that COX-2 expression is determined, in an additive way, by T2D status and IL1-β expression (p-value of the model <0.0001, adjusted $R^2 = 0.45$), but not TLR4 expression. Indeed, in our multi-variable linear regression model, the variable TLR4 was not statistically significant even if it strongly correlated with COX-2 expression. To further assess whether TLR4 could be an important variable, a likelihood ratio test was used to compare the fit of

Table 3 Mean and standard deviation of COX-2, IL1-β and TLR4 normalized gene expression in risk factor subgroups

	COX-2 Mean ± SD			IL1-β Mean ± SD			TLR4 Mean ± SD		
Type 2 diabetes[a]	Yes (16)	No (43)		Yes (16)	No (43)		Yes (16)	No (43)	
	1.67 ± 1.45	0.84 ± 0.72	$p < 0.01$	3.25 ± 2.41	1.04 ± 0.77	$p < 0.01$	1.09 ± 0.42	0.94 ± 0.38	ns
Smoking habit[a]	Yes (22)	No (37)		Yes (22)	No (37)		Yes (22)	No (37)	
	1.57 ± 1.27	0.68 ± 0.53	$p < 0.01$	1.93 ± 1.97	1.20 ± 1.12	ns	1.08 ± 0.41	0.90 ± 0.36	ns
Hypertension[a]	Yes (47)	No (12)		Yes (47)	No (12)		Yes (47)	No (12)	
	1.1 ± 1.05	0.8 ± 0.73	ns	1.64 ± 1.73	1.28 ± 1.18	ns	0.98 ± 0.40	0.96 ± 0.38	ns
Dyslipidemia[a]	Yes (39)	No (20)		Yes (39)	No (20)		Yes (39)	No (20)	
	1.21 ± 1.16	0.74 ± 0.50	ns	1.73 ± 1.84	1.31 ± 1.22	ns	1.20 ± 0.39	0.90 ± 0.39	ns
Sex	M (45)	F (15)		M (45)	F (15)		M (45)	F (15)	
	1.07 ± 1.05	0.96 ± 0.84	ns	1.65 ± 1.75	1.24 ± 1.12	ns	0.92 ± 0.37	0.11 ± 0.43	ns
Age	Beta			Beta			Beta		
	−0.01		ns	−0.007		ns	−0.006		ns

COX-2, IL1-β and TLR4 mRNA values were measured by real-time RT-PCR and normalized to GAPDH (Glyceral dehyde-3-phosphate dehydrogenase) and TBP (TATA box-binding protein). In brackets () the number of subjects for each group

p p-value, *ns* not statistically significant, *sd* standard deviation, *Beta* linear regression coefficient, *ns* not statistically significant (p-value > 0.05)

[a] Missing information for 1 subject

Table 4 Pearson correlation between COX-2 and IL1-β, and COX-2 and TLR4 gene expression considering the degree of two histological features (degree of plaque instability and number of foam cells) in the entire population analyzed

Correlation			COX-2/IL1-β	
Plaque instability			Foam cells	
Grade	R	*p*-value	R	*p*-value
3	0.75	0.030	0.99	0.003
2	0.61	0.001	NA	NA
1	0.68	0.130	0.50	0.046

Correlation			COX-2/TLR4	
Plaque instability			Foam cells	
Grade	R	*p*-value	R	*p*-value
3	0.69	0.030	0.11	0.780
2	0.43	0.009	0.90	0.090
1	0.16	0.560	0.51	0.011

Plaque instability (grade 1 = stable, grade 2 = probably stable, grade 3 = probably unstable), Foam cells = Number of foam cells (grade 1 = none, grade 2 = <50, grade 3 = at least 50)

R Pearson coefficient, *NA* Data not available

two nested models, one with and one without TLR4. The test showed that the presence of TLR4 in the multi-variable model did not influence the level of COX-2 mRNA expression. Thus, the most parsimonious model that describes COX-2 mRNA expression is represented, in an additive way, by T2D status and IL1-β mRNA expression levels. To further investigate whether IL1-β might act as a mediator variable between the risk factor T2D and COX-2 expression, the Baron and Kenny [31] analytic procedure was used. The method showed that expression of COX-2 is influenced by T2D status (57 %) and, to a lesser extent (43 %), by the activation of IL1-β that would act as a mediator variable.

Discussion

COX-2, TLR-4, IL1-β

Atherothrombotic cardiovascular disease is the leading cause of death worldwide despite significant progress in the management of critical risk factors [32]. A major reason for this trend is the ongoing epidemic of obesity-induced insulin resistance and T2D [33]. The mechanisms that promote an inflammatory environment and accelerated atherosclerosis in diabetes are poorly understood. A central regulator of inflammation is NF-κB [34], a transcriptional complex activated by various stimuli including cytokines, oxidized LDL, lipopolysaccharide and oxidant stress [35], all players in atherosclerosis development. In particular, COX-2 is targeted by NF-κB, and its role in inflammation has been demonstrated in several studies [12, 36], including atherosclerosis [16]. Activation of NF-κB by TLR4 signaling and the role of TLR4 in inflammation through a COX-2 dependent pathway have been well documented [24, 37, 38]. Furthermore, recent studies have demonstrated that COX-2 expression may be stimulated by IL1-β in smooth muscle cells [39] and tendon cells [40]. Studies on

animal models reported that TLR4 activation and signaling mediates the synthesis of COX-2 [41, 42] and that TLR4 activation can be induced by hyperglycemia in THP-1 human monocytic cell line [43].

mRNA expression in plaques

Although a strong correlation between COX-2 and TLR4 was observed in T2D patients, TLR4 expression failed to account for COX-2 expression levels in our multi-variable linear regression model and it had to be excluded. However, the Pearson correlation found between COX-2 and TLR4 mRNA expression at foam cells grade 1 could support the hypothesis that the TLR4 receptor is important in the pathogenesis of atherosclerosis by promoting foam cell formation [44]. Activation of TLR4 stimulates mononuclear phagocytes to secrete chemokines which are recognized for their involvement in the recruitment of monocytes and T lymphocytes in the arterial wall [45].

The COX-2 mRNA expression is regulated by a broad spectrum of mediators involved in inflammation, through numerous intracellular pathways, varying between cell types and cellular stimuli [46]. In this study, we observed a significant coordinated overexpression of COX-2 and IL1-β in atherosclerotic plaques of T2D patients and a comparatively weaker coordinated up-regulation in non-T2D patients. Furthermore, the overexpression of COX-2 is significantly increased in T2D patients with a grade 3 plaque instability (both foam cells and overall plaque instability) while it is not statistically significant in grade 1 and 2. This supports the hypothesis that COX-2 may have a different biological role and relevance according to the phase of atherosclerosis progression. IL1-β is also over-expressed in T2D patients with a grade 3 plaque instability (both foam cells and overall plaque instability) enhancing the role of T2D in inducing IL1-β expression as suggested by a previous study [47]. The strong correlation between COX-2 and IL1-β in grade 2 and 3 plaque instability could indicate the progressive involvement of IL1-β in the inflammation process and plaque destabilization. Expressed by multiple cell types in the plaque, particularly foam cells, IL1-β has a negative impact on the health of vascular smooth muscle cells. It leads to the recruitment in the vasculature of monocytes and activated platelets, which secrete IL1-β to form an inflammatory feed-forward loop (auto-stimulation) [48]. A part of histological features were not completely available for all the patients at this time.

A model linking COX-2, IL1-β and T2D in pro-inflammation

The model based on linear regression is consistent with COX-2 overexpression being dependent on T2D status and, to a lesser extent, the mRNA levels of IL1-β mRNA expression. This model would comprise a three-variable system in which two causal paths (the direct impact of the independent variable (T2D) and the impact of IL1-β) feed into the outcome variable, COX-2 mRNA expression. The Baron and Kenny model analysis, which used linear regression coefficients to measure the extent to which T2D (independent variable) influenced COX-2 mRNA expression through IL1-β (mediator), suggests that activation of COX-2 is mediated, at least in part, through an IL1-β dependent pathway. Protein analysis is necessary to evaluate the level of protein expression and it is a potential second step to this work as well as to augment the number

Fig. 1 Comparison of COX-2, TLR4 and IL1-β gene expression between T2D and non-T2D subjects stratifying by grade 3. Overall plaque instability (grade 3 = probably unstable) and grade 3 number of foam cells (grade 3 = at least 50 cells). The other grades and histological features were not statistically significant. *ns* not statistically significant (*p*-value > 0.05), * <0.05, ** <0.01, T2D: subjects with type 2 diabetes
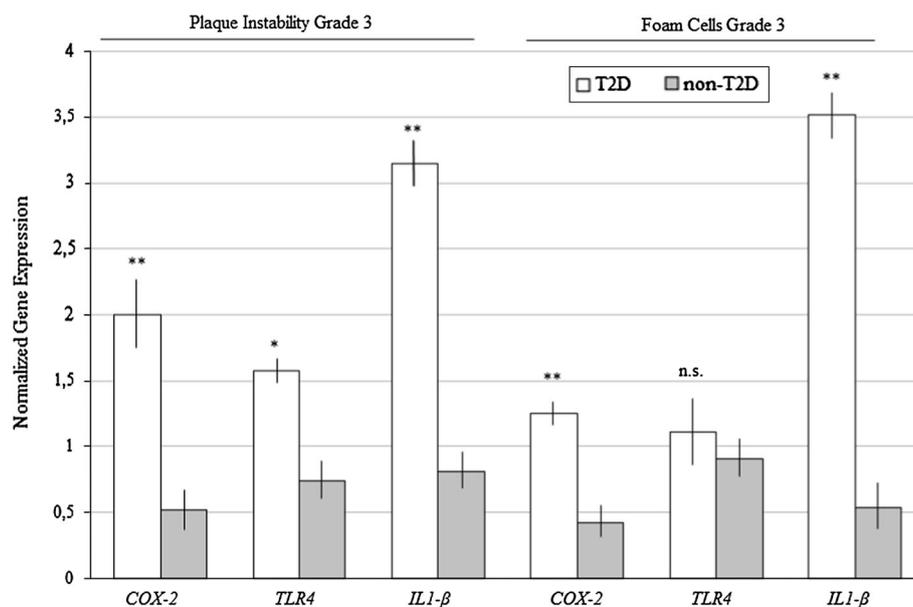
of subjects. Different studies demonstrate how glucose promotes the expression of COX-2 through the activation of IL1-β in mesangial cells [26], and islets of Langerhans [27]. Animal studies suggest that exposure to IL1 promotes atherogenesis; whereas, loss of IL1-β function associates with reduced atherosclerotic lesions [49]. Our findings estimate that the contribution of IL1-β to total COX-2 mRNA expression, would be about 43 % in the plaque of T2D atherosclerotic patients (about 30 % in non-T2D patients). Like T2D, type 1 diabetes (T1D) is a risk factor linked to atherogenesis through the production of prosta-glandins in a COX-mediated inflammation [50]. T1D leads to an inflammatory process in which there is a significant increase of cytokines (IL-1, IL-6, IL-18, and TNF-α) [51, 52]. Direct blockade of IL1-β has been studied extensively as a therapeutic strategy for T1D at the preclinical level [53]. Our results indicate that it is worth investigating whether anti-inflammatory agents targeting IL-1β could decrease significantly COX-2 mRNA expression levels; and thus, diminish the augmented inflammatory response. In turn, a diminished inflammatory response may reduce the probability of plaque disruption and, consequently, the risk of ischemic stroke.

Conclusions

Our results support the hypothesis that T2D pathology contributes in vivo to increase the inflammatory process associated with the atherosclerotic plaque formation, as shown by an increment of COX-2 and IL1-β mRNA expression.

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