Review Article

Prevention of cardiovascular disease in type-2 diabetes: How to improve the clinical efficacy of aspirin

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Summary

Atherosclerotic cardiovascular disease and its thrombotic complications are the principal causes of morbidity and mortality in patients with type-2 diabetes. Aspirin reduces the risk of thrombotic events in a broad range of patients with vascular disease and, in selected individuals, is beneficial for primary prevention. Although recommended by existing guidelines, in secondary and in primary prevention trials the clinical efficacy of low-dose aspirin in patients with diabetes appears to be substantially lower than in individuals without diabetes. In this review, we discuss possible mechanisms that may contribute to reduce the antithrombotic activity of aspirin in diabetes. We also discuss adjuvant therapies used in diabetic patients that may potentially improve the antithrombotic efficacy of aspirin.

The association between diabetes and cardiovascular (CV) disease and its thrombotic sequelae is well established. Macrovascular complications are the principal causes of morbidity and mortality in patients with type-2 diabetes. In addition to classical cardiovascular risk factors such as dyslipidemia, obesity and hypertension, which are more common in diabetic patients, other metabolic abnormalities such as hyperglycemia, hyperinsulinism and insulin resistance, advanced glycosilated end products (AGEs) and increased oxidative stress characterize diabetes. All these factors likely provoke molecular events in endothelial cells and in circulating platelets and leukocytes that contribute to the development and progression of atherosclerosis and increase cardiovascular risk in diabetic patients (1). Optimal clinical management of vascular disease in diabetes remains to be established. While the link between microvascular complications and hyperglycemia has been clearly established, the effect of intensive pharmacological treatment for metabolic control on macrovascular complications is less evident (2). Furthermore, a more than two-fold increased risk of CV events is already present in subjects with impaired glucose tolerance (IGT), that is normal fasting blood glucose levels and elevated values after oral glucose load (3). Thus additional therapeutic approaches appear to be needed to reduce the high cardiovascular risk associated with impaired glucose metabolism.

The clinical evidence of lower efficacy of aspirin in diabetic patients

Aspirin reduces the risk of events in a broad range of patients with vascular disease in secondary prevention trials and in selected individuals in primary prevention trials. However, not all of the population at risk is protected, and the existence of 'aspirin non-responders' has been advocated as a possible explanation for these findings (4–7). The use of low-dose aspirin in subjects with diabetes is recommended by existing guidelines (8–9). Despite the general consensus, the evidence supporting these recommendations is surprisingly scant. The only evidence to support the efficacy of aspirin in the primary prevention of cardiovascular disease (CVD) in diabetic patients comes from the U.S. Physicians' Health Study, in which a 60% reduction in the risk of myocardial infarction was noted (10). This risk reduction was not statis-

tically significant, due to the small number of events (11/275 in the aspirin group vs. 26/258 in the placebo group; p=0.22). Furthermore, only male physicians were enrolled in the early eighties, when other effective strategies for CVD prevention (e.g. ACE inhibitors and statins) were not yet available. Recent observations indicate that diabetic patients receive lower cardioprotective benefit from aspirin than non-diabetic ones. In fact, a meta-analysis on the efficacy of antiplatelet therapy in the prevention of major cardiovascular events showed a clear benefit for the whole population of over 140,000 subjects (22% reduction in the risk of major CV events), but no statistically significant benefit in the subgroup of about 5,000 diabetic patients (7% risk reduction) (11). Within the meta-analysis, the Early Treatment Diabetic Retinopathy Study (ETDRS) trial was the only one specifically conducted in diabetic patients. In this trial, treatment with a daily dose of 650 mg of aspirin for an average of 5 years was associated with a non-significant 9% reduction in serious vascular events (vascular death, non-fatal MI, non-fatal stroke) in 3,711 diabetic patients with and without previous CVD (12).

More recently, a subgroup analysis of over 1,000 diabetic patients in the Primary Prevention Project (PPP) (13) showed that low-dose aspirin only marginally reduced the risk of major CV events after three years of follow-up (relative risk = 0.90; 95% CI 0.50–1.62) (14). Though not conclusive, these results suggest that the diabetic patient may represent a special case of aspirin non-responder, although, to our knowledge, no specific studies have fully explored this hypothesis.

Diabetes is also often associated with other cardiovascular risk factors, particularly hypercholesterolemia. The presence of elevated values of total cholesterol was associated with lower benefit from aspirin in the Physicians' Health Study (10) and the Thrombosis Prevention Trial (15).

Reasons for aspirin therapeutic failure

The significance of the therapeutic failure of aspirin in cardiovascular disease remains controversial due to the lack of standardized definitions, detection methods, and clinical studies (16–18). However, recent studies indicated that the laboratory evidence of reduced aspirin responsiveness, defined either by inadequate inhibition of platelet function *in vitro* (7) or by inadequate suppression of thromboxane A_2 (TxA_2) production *in vivo* (6), may identify patients who will experience cardiovascular events, on aspirin treatment, at a higher rate than aspirin-sensitive patients.

Poor compliance or insufficient dosing may reduce the effectiveness of aspirin. However, these factors are unlikely to have influenced results in clinical trials. First, compliance is usually more controlled in clinical trials than in normal practice; second, high-dose aspirin appears to be no more effective than low-dose in diabetic individuals. The ETDRS and the PPP trials yielded similar results, despite use of 650 mg aspirin in the former (12), and low-dose aspirin in the latter (14). Thus, it seems reasonable that intrinsic mechanisms of action of the drug accounts for its lower efficacy in individuals with diabetes.

Two main factors intrinsic to the mechanism of action of aspirin may be responsible for its failure to produce the expected pharmacological effect in diabetic patients:
1. a pro-inflammatory, pro-thrombotic environment conducive to platelet activation and thrombus formation through pathways that no longer require either platelet-activation amplification loops or the vasoconstriction mediated by TxA_2, the metabolic product of the aspirin target cycloxygenase-1 (COX-1);
2. failure of aspirin to adequately suppress TxA_2 production.

Determinants of the thrombogenic environment in diabetes

The role of endothelial dysfunction and inflammation

Endothelial dysfunction and inflammation may play a key role in the initiation and progression of diabetic macrovascular disease (1). Diabetic macrovascular disease is characterized by accelerated progression of atherosclerosis (19) accompanied by a greater incidence of complications such as plaque ulceration, fissuration and thrombosis. More extensive macrophage invasion and inflammation appears to occur in atherosclerotic plaques of diabetic patients as compared with non-diabetic ones (20). Inflammatory marker predictors of cardiovascular risk, such as C-reactive protein and interleukin-6 (21, 22), are elevated in diabetic patients (23, 24). C-reactive protein may induce an inflammatory-thrombogenic phenotype in endothelial cells by up-regulating the expression of adhesive molecules (25), and down-regulating nitric oxide (NO) synthase transcription and NO release (26). Interestingly, these effects of C-reactive protein are significantly increased by high glucose concentrations *in vitro* (27). Inflammatory markers have also been shown to be predictors of type-2 diabetes development (28), strengthening the concept that sub-clinical vascular inflammation is part of a pre-diabetic condition. Circulating levels of the proinflammatory cytokine, CD40 ligand (CD40L), are elevated in diabetic patients and reduced following thiazolidinedione treatment (29, 30). CD40-CD40L signaling in endothelial cells and in monocyte-macrophages mediates a broad range of pro-atherogenic functions (31), including generation of reactive oxygen species (ROS) generation and expression of adhesive molecules, macrophage chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) (32–34). The bulk of soluble CD40L in circulation derives from activated platelets. In hypercholesterolemic mice, the disruption of CD40 signaling reduces the progression of atherosclerosis (35). Upregulation of CD40L in circulation suggests platelet activation and pro-inflammatory, pro-atherosclerotic state in diabetic patients. CD40L induces tissue factor (TF) expression on monocytes and contributes to stabilize platelet arterial thrombi in *in vivo* injury models (36).

Several factors specific for the diabetic condition, including hyperglycemia, hyperinsulinemia and insulin resistance, increased oxidative stress and AGEs, may induce endothelial dysfunction (Fig. 1). An impaired endothelium-dependent vasodilation, due to a reduced release of endothelium-derived relaxing factors including NO and prostacyclin, has been demonstrated in animal models of diabetes and in humans (37, 38). Experimental evidence indicates that hyperglycemia, hyperinsulinemia, increased oxidative stress and AGEs may directly upregulate a variety of humoral and cellular inflammatory reactions (39), which play an important role in atherothrombosis (40). In particular, increased expression of leukocyte adhesive molecules occurs in endothelial cells exposed to high glucose concentrations *in vitro*
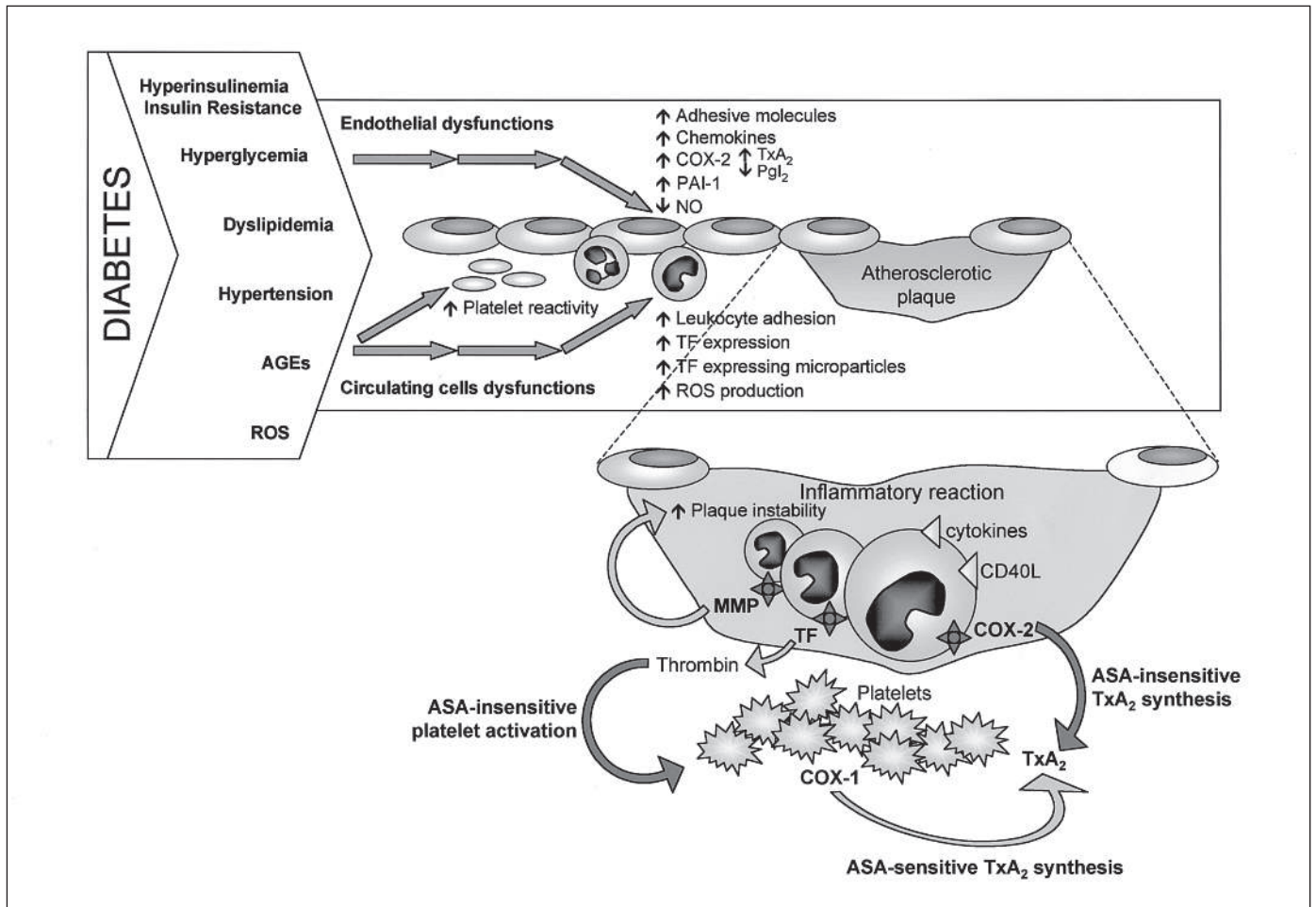
Figure 1: **Patho-physiological pathways potentially implied in the reduced clinical efficacy of aspirin in diabetes.**

(41), and acute hyperglycemia increases circulating plasma levels of soluble ICAM-1 in normal subjects (42). In accordance with these observations, circulating levels of different endothelial-derived adhesive molecules are increased in diabetic patients (42–47). Not only high glucose concentrations *per se,* but also AGEs, which are increased in diabetic patients, induce a pro-inflammatory phenotype in endothelial cells, including upregulation of adhesive molecules (37, 48). Circulating monocytes and neutrophils also undergo rapid upregulation of adhesive molecules after acute hyperglycemia in both normal subjects and type-2 diabetic patients (49). *In vitro*, high glucose concentrations induce the expression of a variety of proinflammatory cytokines and β2-integrins in monocytic cells (50) and increase monocyte and neutrophil adhesion to endothelial cells (41, 51).

A number of observations indicate that diabetes is associated with an upregulation of circulating thrombogenic factors. Many studies suggest that platelet function is upregulated (52, 53) and that the balance between procoagulant and profibrinolytic factors is altered in diabetes. Increased levels of von Willebrand factor, factors VII and VIII and plasminogen activator inhibitor-1 (PAI-1) (39, 54–56) have all been reported. Furthermore, leukocytes are activated and release increased amounts of ROS (57, 58). Increased expression of tissue factor, the major monocyte-macrophages-associated procoagulant activity, has been re-

ported in patients with diabetes (59–61). Circulating cell membrane fragments carrying procoagulant activity, the so-called microparticles, are also elevated (56, 59). Higher numbers of tissue factor exposing, leukocyte-derived, circulating microparticles correlate with the metabolic syndrome in type-2 diabetic patients without clinical evidence of vascular disease (61). Upregulation of tissue factor expression by leukocytes may contribute to formation of thrombin and increased thrombogenicity observed in diabetic patients by Rauch et al. (62).

The role of platelets
Platelets play a key role in atherothrombosis, and an impaired platelet response to the inhibitory effect of aspirin has been used as a marker to define 'aspirin resistance' (7).

Pioneering studies demonstrated elevated levels of urinary metabolites of TxA_2 in diabetic patients, which were attributed to increased platelet activation *in vivo* (63, 64).

A number of experimental studies, recently reviewed by Vinik et al (65), indicate the existence of functional abnormalities in platelets from diabetic patients. In particular, early work showed an increased aggregability of platelets from individuals with diabetes. This response was attributed to an increased activity of the TxA_2 synthase system (66), but this observation was not confirmed by others (67). Moreover, an increased response

of diabetic platelets to different agonists has not been consistently demonstrated (68, 69). More recently, Hu et al reported increased platelet and leukocyte activation and interaction in type-1 diabetes with microangiopathy (70).

With regard to insulin effect on platelet function, previous studies have yielded complex results. Insulin may act as negative regulator of platelet function and reduces platelet response to different agents (71). Interestingly, this effect of insulin is absent in obese individuals as well as in obese type-2 diabetic patients (72, 73), suggesting that insulin resistance may also occur in platelets and may be responsible for an increased platelet reactivity. In contrast, other studies showed platelet activation, rather than inhibition by insulin. For example, insulin has been reported to enhance platelet fibrinogen binding in type-1 diabetic patients (74). It has been suggested that insulin may exert an opposite effect on platelets at physiological and supraphysiological concentrations (75). This also suggests that platelet abnormalities may be different in type-1 and type-2 diabetes.

Besides insulin resistance, hyperlipidemia seems to be associated with an increased platelet response *in vitro* (76) and *in vivo* (77). Although previous studies failed to detect an increased platelet aggregation response in patients with hyperlipoproteinemia (78), it was recently reported that patients with cardiovascular disease, who show poor platelet responsiveness to aspirin in *in vitro* aggregation assay, have significantly higher concentrations of total and LDL cholesterol than patients whose platelets are responsive to aspirin (79).

Gum et al (80) recently investigated the incidence and clinical predictors of 'aspirin resistance', defined as reduced platelet sensitivity to the anti-aggregating effect of aspirin, in a cohort of 325 patients with stable cardiovascular disease who were receiving aspirin (325 mg/day). In this group of patients, 5.5% were found to be aspirin non-responders. However, there was no difference in aspirin sensitivity between diabetic and non-diabetic patients. The low number of diabetic patients in this study does not allow definitive conclusions; however, on the basis of these data, it appears that the evidence of reduced aspirin responsiveness by platelet function tests does not necessarily correlate with clinical evidence of the aspirin failure, as suggested by inadequate protection of diabetic patients from cardiovascular events. In contrast, a more recent study documented a reduced response to aspirin of platelets from type-2 diabetic patients compared with platelets from healthy subjects. The reduced response to aspirin in diabetic patients was associated with poor metabolic control (81). Therefore, an intrinsic difference in sensitivity to aspirin of platelets seems not to completely explain the reduced anti-thrombotic efficacy of aspirin in diabetic patients. Rather, cross talk between circulating inflammatory mediators, the vessel wall and platelets may contribute to the failure of aspirin to adequately prevent cardiovascular events in diabetic patients (Fig. 1) (82).

Inadequate suppression of TxA$_2$-production

Pathways of aspirin-insensitive TxA$_2$ synthesis
Pharmacological failure of aspirin could also involve TxA$_2$ production *via* aspirin-insensitive eicosanoid biosynthetic pathways (83), that is from sources other than platelet COX-1. COX-2, an inducible enzyme mainly expressed in monocyte-macrophages exposed to inflammatory stimuli, is a major candidate as an aspirin-insensitive source of TxA$_2$. Interestingly, monocytes isolated from diabetic patients show an increased ability to produce TxA$_2$ following stimulation *in vitro* (59). The exposure of cultured endothelial cells to high glucose concentrations results in upregulation of COX-2, down-regulation of NO synthase, and alterations in the balance of prostanoid synthesis in favor of TxA$_2$ with respect to PGI$_2$ (84) (Fig. 1).

It has been suggested that aspirin-insensitive COX-2 is an additional source of TxA$_2$ in platelets (85). Although COX-2 can be found in platelets under pathological conditions (86), the metabolic relevance of this enzyme in platelets remains elusive (86, 87). No data are available on the expression of COX-2 in platelets from individuals with diabetes.

COX-1 isoforms insensitive to aspirin
Although a number of single nucleotide polymorphisms in the gene encoding for COX-1 exists (88), there is no evidence that genetic variations of COX-1 make the enzyme resistant to aspirin or that single nucleotide mutations in the COX-1 gene are more frequent in diabetes.

Single nucleotide polymorphisms in the gene encoding for the β3 chain of the fibrinogen receptor have been suggested to be associated with a reduced anti-platelet efficacy of aspirin (89); however, there are no data suggesting that these polymorphisms are more highly represented in diabetes.

In summary, many lines of evidence indicate that the pro-inflammatory and pro-thrombotic state that accompanies diabetes may create a milieu in which complete blockade of COX-1 by aspirin does not modify the overall platelet response, and has little effect on thrombus formation. The inflammatory state may also contribute to aspirin-insensitive TxA$_2$ synthesis, *via* an increased COX-2 activity.

It is, thus, plausible that pharmacological interventions capable of modifying the inflammatory burden associated with diabetes may improve the efficacy of aspirin.

How to modify the inflammatory burden

Increasing evidence indicates that targeting traditional risk factors such as hypercholesterolemia, hypertension, and insulin resistance using HMG-CoA reductase inhibitors (statins) and fibrates, angiotensin converting enzyme (ACE) inhibitors and β–blockers, and thiazolidinediones (TZD), results in a significant improvement in a number of circulating markers of inflammation and endothelial dysfunction. Recent experimental data indicate that through pleiotropic actions, some classes of these drugs may also directly affect molecular pathways of inflammation, which have been implicated in the pathogenesis, progression, and complications of both atherosclerosis and diabetes.

Here we will focus our attention on statins, ACE inhibitors and TZDs. Indeed, in recent years a substantial amount of clinical and experimental data has accumulated suggesting that these classes of drugs exert effects on multiple targets, to reduce inflammatory events potentially involved in atherosclerosis and aspirin resistance (90–92) (Fig. 2).
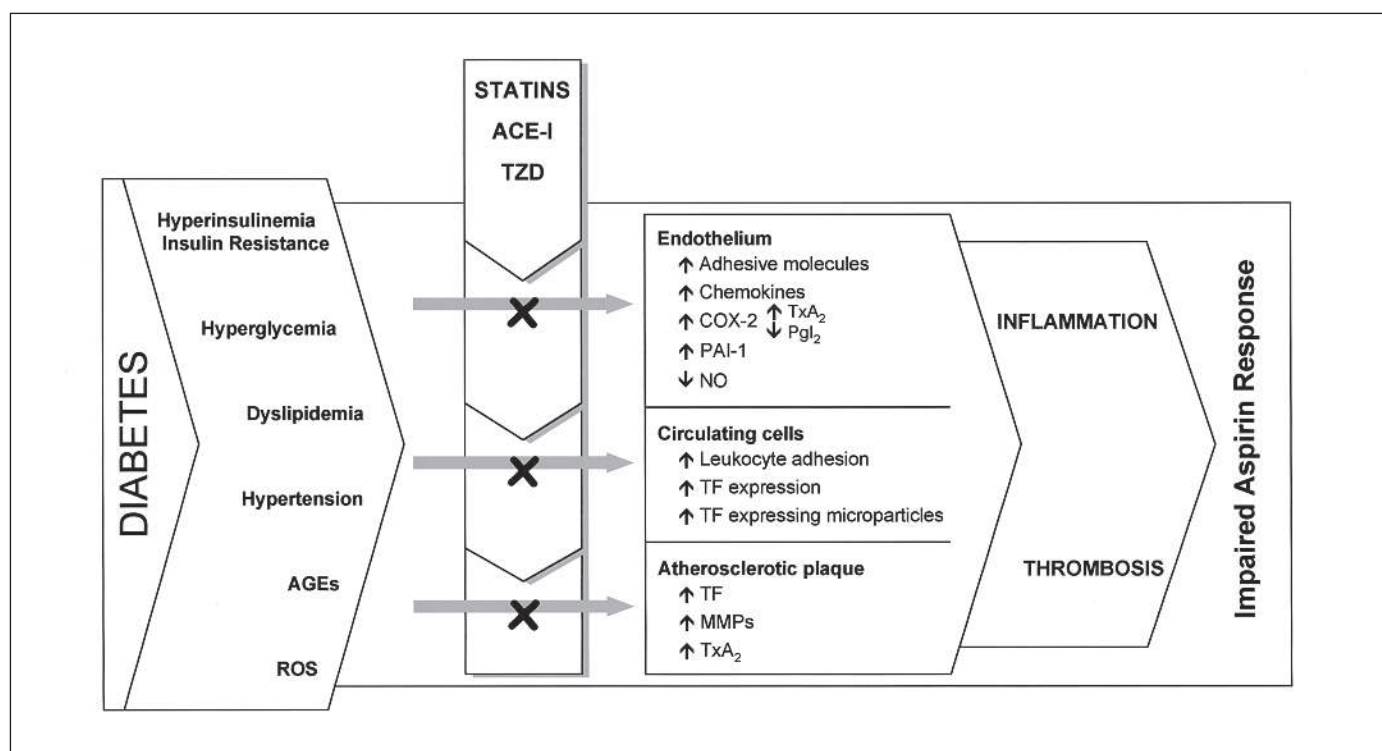
Figure 2: **Mechanisms of action of statins, ACE-inhibitors and Thiazolidinediones that could interfere with the pathways leading to a reduced clinical efficacy of aspirin.**

Statins

The beneficial effects of statins in the primary and secondary prevention of coronary heart disease have been demonstrated in several large-scale clinical trials (93). Recent evidence suggests that the clinical benefits obtained with statin therapy may extend beyond their effect on blood cholesterol levels and may involve a potential anti-inflammatory effect (94–96). A large body of experimental studies supports the concept that the putative anti-inflammatory effect of statins is mediated by the inhibition of a variety of inflammatory-thrombogenic functions in leukocytes and improvement of endothelial dysfunctions (90). The sites of plaque rupture are often the sites of inflammatory reaction. Activated inflammatory cells at these sites (97) are the major cellular source of matrix metalloproteases, which contribute to the proteolytic activity, TF, that induces the formation of thrombin, and COX-2, which may be the source of aspirin insensitive eicosanoids (98). In experimental models in primates, statin therapy results in a significant reduction in levels of VCAM-1 expression on the endothelium, macrophage infiltration and interleukin-1β and tissue factor in atherosclerotic lesions (99).

Statins are potent inhibitors of the expression and function of adhesion molecules in leukocytes (100), thus reducing leukocyte adhesion to endothelial cells *in vitro* (101), as well as leukocyte recruitment in animal models (102). Statins also increase the expression of endothelial NO synthase (103), and reduce TF expression in human monocytes (104) and in cultured endothelial cells (105). The biochemical effects underlying such a wide variety of cellular effects of statins remain to be fully clarified. By inhibiting L-mevalonic acid synthesis, statins affect not only the production of cholesterol, but also that of other isoprenoids such as farnesylpyrophosphate and geranylgeranylpyrophosphate. The covalent binding of these lipids to a variety of cell signaling proteins allows their proper subcellular localization and function. Among these proteins, small GTP-binding proteins of the Rho family have been consistently implicated to explain the inhibitory effects on inflammatory cell function, including: leukocyte-endothelial cell adhesion (101), endothelial NO synthase expression (103), TF expression in human monocytes (104) as well as in cultured endothelial cells (105) *in vitro*. By reducing the inflammatory reaction in atherosclerotic lesions, statins treatment may improve plaque stability in humans (106).

Clinical trials have shown that statins reduce cardiovascular events in individuals with diabetes (107). Similarly to non-diabetic patients, the beneficial effect of statins on dyslipidemia is flanked by positive effects on vascular function and markers of systemic inflammation in diabetic patients (108), suggesting that these drugs may also improve vascular response in the atherothrombotic context of diabetes. If an upregulated vascular inflammatory reaction is responsible for aspirin resistance, it is plausible to hypothesize that statins would also improve the clinical response to aspirin therapy.

ACE inhibitors

There is increasing evidence that ACE inhibitor therapy reduces cardiovascular risk in people with diabetes (109, 110). A number of experimental studies in animal models and humans have shown that ACE inhibition significantly reduces endothelial dysfunction associated with atherosclerosis (111). Moreover, angiotensin II, the AT1 receptor, and ACE are expressed in macrophages at the shoulder region of coronary atherosclerotic plaque

from patients with unstable angina (112), as well as in endothelial cells, vascular smooth muscle cells (VSMC), and immune cells of carotid atherosclerotic lesions (113), suggesting a possible role of ACE in the development and progression of atherosclerosis. In fact, angiotensin II induces the synthesis and release of IL-6, which co-localizes with angiotensin II, AT1 receptor, and ACE in macrophages in coronary atherosclerotic plaque (112). In agreement with these observations, in a placebo-controlled trial, Soejima et al found that treatment with ACE inhibitors reduced circulating levels of MCP-1 and TF in patients who had had myocardial infarction (114). In fact, *in vitro* TF synthesis in monocytes and endothelial cells stimulated by endotoxin or inflammatory cytokines is enhanced by angiotensin II, and, conversely, inhibited by competitive inhibitors of its receptor AT1 (115–117), indicating that ACE inhibition may prevent the increased thrombogenic potential mediated by TF at the site of atherosclerotic lesions.

Thiazolidinediones (TZDs)

Although initially identified as important regulators of metabolic processes in adipocytes and hepatocytes (118), recent experimental data established that activation of Peroxisome Proliferation Activated Receptors (PPARs) by natural or pharmacological ligands may switch off a number of inflammatory responses in endothelial cells, VSMC, circulating monocytes as well as in macrophages at the site of atherosclerotic plaque (119).

In particular, TZDs, pharmacological PPARγ agonist, have been shown to inhibit VSMC proliferation and migration, an important event of the vascular response to injury, *in vitro* (120) and *in vivo* (121), and inhibit the production of inflammatory cytokines by monocyte-macrophages *in vitro* (122, 123). Moreover, troglitazone reduces the expression of adhesive molecules in cultured endothelial cells and the adhesion of leukocytes (124, 125). This effect was also observed with pioglitazone, which decreased monocyte adhesion to endothelial cells under flow, possibly through a modulation of the small GTPase, RhoA, or focal adhesion kinase activity (126).

Treatment with troglitazone also reduced monocyte-macrophages homing in atheroscletic plaques of apoE-deficient mice (125) and inhibited the development of atherosclerosis in LDL-receptor-deficient mice (127). Troglitazone showed an effect on the development of atherosclerosis in WHHL rabbits comparable to that of pravastatin; interestingly, the two drugs showed a synergistic effect (128). These data in experimental models have been supported by a study in type-2 diabetic patients showing that TZD treatment is associated with reduced carotid atherosclerotic lesions (129). More recently, the anti-inflammatory effects of TZDs have been further supported by the evidence that these drugs reduce a number of inflammatory markers in diabetic patients. Treatment with troglitazone decreased by 60% circulating levels of C-reactive protein (23) and rosiglitazone significantly reduced circulating levels of C-reactive protein, metalloprotease-9 (MMP-9), and leukocytes in type-2 diabetic patients (24). In the same type of patients, both rosiglitazone and troglitazone significantly reduced circulating levels of CD40L

(26, 27), an important pro-inflammatory mediator playing a role in the development of atherosclerosis (28). Despite platelets being anucleated cells, PPARγ agonists have been shown to reduce platelet activation both *in vitro* (130, 131) and *in vivo* in cardiovascular patients without diabetes (132). The unexpected anti-platelet activity of TZD has been recently clarified by the discovery of functional PPARγ in platelets and in megakaryocytes (133). Thus, like statins, TZD might improve the clinical response of diabetic patients to aspirin, by reducing the inflammatory burden of diabetic atherosclerosis and inhibiting platelet response.

Conclusions

On the basis of the existing data it is reasonable to hypothesize that:

1. an upregulated vascular inflammatory-thrombogenic state is responsible for both the increased cardiovascular risk and the "suspected" clinical inefficacy of aspirin in diabetic patients;
2. different classes of drugs, including statins, ACE inhibitors, and TZD, with documented effects on oxidative stress and nitric oxide metabolism, coagulation, inflammation and adhesion of immune cells to the vascular endothelium, can represent hypothetical candidates to improve the anti-thrombotic efficacy of aspirin in diabetic patients.

These hypotheses remain to be investigated at the laboratory bench and, even more urgently, in randomized clinical trials. The latter should test the effects of low-dose aspirin in populations of adequate sample size, with a strict control of glucose metabolism and cardiovascular risk factors. The use of a factorial design, including statins, the class of drugs for which pleiotropic effects have been more extensively described, could help to clarify whether aspirin efficacy could be improved by the concomitant use of other drugs with anti-inflammatory properties.

The results of these studies would greatly improve our knowledge about the effectiveness of existing strategies for the prevention and treatment of cardiovascular complications in diabetic patients.

Acknowledgement

Abbreviations

AGEs = Advanced glycosilation end-products; ROS = Reactive oxygen species; COX = Cycloxygenase; TxA_2 = Tromboxane A_2; PgI_2 = Prostacycline I_2; PAI-1 = Plasminogen activator inhibitor-1; NO = Nitric Oxide; TF = Tissue factor; MMP = Matrix metallo proteinases.

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