Decreased expression of γ-carboxylase in diabetes-associated arterial stiffness: impact on matrix Gla protein

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Aims
Arterial stiffness is accelerated in type 1 diabetic patients. Medial artery calcification (MAC) contributes to the development of arterial stiffness. Vitamin K oxidoreductase (VKOR) reduces the vitamin K required by γ-carboxylase to activate matrix γ-carboxyglutamic acid (Gla) protein (MGP), an inhibitor of vascular calcification. This study aimed to evaluate the hypothesis that diabetes reduces the γ-carboxylation of MGP in the aortic wall, leading to increased vascular calcification, and the role of γ-carboxylase and VKOR in this γ-carboxylation deficit.

Methods and results
Type 1 diabetes was induced in male Wistar rats with a single ip injection of streptozotocin. Augmentation of arterial stiffness in diabetic rats was shown by a 44% increase in aortic pulse wave velocity. Aortic and femoral calcification were increased by 26 and 56%, respectively. γ-Carboxylated MGP (cMGP, active) was reduced by 36% and the aortic expression of γ-carboxylase was reduced by 58%. Expression of γ-carboxylase correlated with cMGP (r = 0.59) and aortic calcification (r = −0.57). VKOR aortic expression and activity were not modified by diabetes. Vitamin K plasma concentrations were increased by 191% in diabetic rats. In ex vivo experiments with aortic rings, vitamin K supplementation prevented the glucose-induced decrease in γ-carboxylate expression.

Conclusion
Our results suggest that reduced cMGP, through an impaired expression of γ-carboxylase, is involved in the early development of MAC in diabetes, and therefore, in the acceleration of arterial stiffness. A defect in vitamin K uptake by target cells could also be involved.

Keywords
Matrix Gla protein • γ-Carboxylase • VKOR • Diabetes • Arterial stiffness

1. Introduction
Elastic properties of conduit arteries assure their primary function of dampening the pulsatile blood flow generated by the heart. However, ageing causes degenerative changes in the mechanical properties of the vessel wall of large arteries, leading to their stiffening. Stiffer arteries have a reduced compliance, resulting in a faster pressure wave propagation along the arterial tree and therefore, early wave reflection. Arterial stiffness is responsible for isolated systolic hypertension and is associated with left ventricular hypertrophy, coronary heart disease, and chronic kidney disease. Arterial stiffness has also been identified as an independent predictor of cardiovascular risk in the normo- and hypertensive population and consequently, has become an important therapeutic target.

Accelerated arterial stiffness in type 1 diabetes mellitus (T1DM) is supported by a large body of evidence. In fact, arterial stiffness occurs an average of 15 years earlier in patients with T1DM compared with non-diabetic subjects, as demonstrated by the premature rise in pulse pressure (PP) seen in this population. Thus, it is suggested that at least part of the increased cardiovascular risk associated with diabetes may be due to arterial stiffness. Medial artery calcification (MAC), characterized by the deposition of hydroxyapatite crystals along the elastic lamellae of large arteries and subsequent fragmentation of the elastic network, contributes to arterial stiffness. MAC is highly prevalent among T1DM patients and is a risk marker for cardiovascular events in this population. Matrix γ-carboxyglutamic acid (Gla) protein (MGP) is a vitamin K-dependent protein (VKD), whose role as an inhibitor of vascular calcification, and results...
calciﬁcation has been highlighted by the extensive MAC observed in MGP knockout mice and in warfarin-treated rats. Total MGP mRNA was reported to be lower in peripheral arteries of diabetic patients with MAC, implicating MGP in the regulated process of MAC. MGP is post-translationally modiﬁed by γ-carboxylation, an integral protein of the endoplasmic reticulum that uses the energy of vitamin K hydroquinone oxygenation to convert glutamic acid residues into Ca2+-binding Gla to activate MGP and other VKD proteins. The vitamin K oxidoreductase (VKOR) then reduces the vitamin K epoxide product back to vitamin K hydroquinone to complete the cycle. Hence, γ-carboxylation together with VKOR is expected to play crucial roles in allowing proper MGP function. Immunofluorescence staining showed that MGP colocalized with calciﬁcation areas in its undercarboxylated form (ucMGP, inactive) and it was suggested that this accumulation of ucMGP could be related to impaired γ-carboxylation and to the loss of protection against calciﬁcation. However, the presence of ucMGP has only been evaluated locally at sites of overt calciﬁcation, and the mechanisms involved in γ-carboxylation impairment of MGP have not been investigated yet.

The aim of this study was to test the hypothesis that diabetes reduces γ-carboxylated MGP (cMGP, active) in the vessel wall early in the disease, contributing to the development of MAC and consequently, the acceleration of arterial stiffness. We hypothesized that the γ-carboxylation deﬁcit was attributable to a deﬁcient γ-carboxylase and/or an inadequate recycling of vitamin K by VKOR. A rat model of diabetes-associated arterial stiffness was used to investigate the modulation of γ-carboxylation and VKOR in the context of diabetes and the impact of this modulation on the γ-carboxylation of MGP.

2. Methods

2.1 Animal procedures

A total of 60 male Wistar rats aged 5 weeks (Charles River Laboratories, QC, Canada) were used in this study. Animals were housed for 1 week before the beginning of the study, and had free access to water and chow. After an overnight fast, rats (n = 30) were administered a single ip injection of streptozotocin (STZ) (60 mg/kg) freshly diluted in 0.05 mol/L citrate buffer at pH 4.5. Blood glucose levels were monitored every other day with a glucometer (Accusoft Advantage from Roche, QC, Canada) and animals with blood glucose levels > 250 mg/dL were considered diabetic. Control rats (n = 30) consisted of age-matched untreated rats. Rats were sacriﬁced after 9 weeks. Food was removed 12 h before sacriﬁce, and rats were anaesthetized with an ip injection of sodium pentobarbital (65 mg/kg). The depth of anaesthesia was evaluated by the foot-to-foot method. Hence, γ-carboxylation and systolic blood pressures at each site. Pulse wave velocity (PWV) were connected to a pressure transducer for measurements of diastolic and systolic blood pressures at each site. 

2.2 Vascular calciﬁcation

Sections of the thoracic aorta and the femoral artery were dried for 1 h at 55°C. Calcium was extracted with 10% formic acid (30 μL/mg) overnight at 4°C. The colorimetric quantiﬁcation of the calcium content was achieved following the reaction of calcium with o- cresolphthalein in an alkaline medium (Teco Diagnostics, CA, USA). Results are expressed as μg of calcium per mg tissue.

2.3 Collagen content

Five micron aortic sections were stained with Masson’s Trichrome according to the manufacturer’s instructions (Sigma-Aldrich, ON, Canada). For quantiﬁcation, sample images (four images per aorta, 400 × magnification) were imported in Visiopharm Integrator System (3.4.1.0) Micromager. Using the acquisition module, a region of interest was deﬁned to outline the media in each image. Quantitative analysis of the collagen area using a Bayesian classiﬁcation method was applied on all the samples. Results are expressed as % of the collagen area in aortic media.

2.4 Immunoblotting

A portion of the pulverized aorta was lysed in extraction buffer. Equal amounts of protein (50 μg) were loaded onto either a glycine- γ-carboxylase or a tricine-sodium dodecyl sulfate polyacrylamide gel (VKOR and cMGP) as described in Schagger and von Jagow and transferred to a BioTrace NT (Pall Corporation, FL, USA) nitrocellulose membrane after electrophoresis. Membranes were hybridized with primary antibodies directed against VKOR (Acris Antibodies, CA, USA), γ-carboxylase (Abcam, Inc., MA, USA), cMGP (Enzo Life Sciences, NY, USA) or GAPDH (Cell signaling, MA, USA) followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit, Cell Signaling). Membranes were incubated with ECL Plus (GE Healthcare Life Sciences, NJ, USA), exposed to ﬁlm and bands were quantiﬁed by densitometry. Results are normalized to GAPDH.

2.5 Immunofluorescence

Parafﬁn-embedded aortic segments were cut into 5 μm sections, deparafﬁnized, and incubated with a mouse monoclonal antibody against ucMGP (Enzo Life Sciences), then with a goat anti-mouse secondary antibody coupled to Alexa Fluor546 (Invitrogen, OR, USA) and counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen). Substitution of primary antibody with PBS was used as a negative control and aortic sections of rats treated with warfarin were used as a positive control. Aortic sections were visualized with a ﬂuorescence microscope and photomicrographs were captured at ×100 and ×400 magniﬁcation.
2.6 Aortic microsomes

Aortic samples from seven to eight rats were pooled to yield one microsome preparation. Therefore, four microsomal preparations per group were obtained. Details of the protocol are provided in the Supplementary material online. Microsomes were stored at −80°C for <1 week before use.

2.7 VKOR enzymatic activity

The enzymatic activity of VKOR was assayed in aortic microsomes by measuring the formation of vitamin K from vitamin K epoxide. Details of the protocol are provided in the Supplementary material online. Results are expressed as nanomole of vitamin K per milligram of microsomal protein.

2.8 Vitamin K plasma concentrations

For vitamin K quantification, samples were prepared as described in Fu et al., with minor modifications. Details of the protocol are provided in the Supplementary material online. Results are expressed as nanogram of vitamin K per millilitre of plasma.

2.9 Osteocalcin plasma concentrations

Undercarboxylated (ucOC) and γ-carboxylated osteocalcin (cOC) concentrations were measured with rat competitive enzyme immunoassay kits, both from Takara (Takara Bio, Inc., Otsu, Shiga, Japan) and according to the manufacturer’s instructions. Results are expressed as ng of OC/mL of plasma.

2.10 Ex vivo experiments

A total of 12 male Wistar rats weighing 375–400 g were sacrificed as described above. The thoracic aorta was isolated under sterile conditions and cleaned of perivascular fat and connective tissue. Segments (~4–5 mm) were randomized and placed in DMEM (Wisent, Inc., QC, Canada) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and containing either a normal (NG, 5 mM) or a high concentration of glucose (HG, 25 mM). As an additional condition, HG was supplemented with vitamin K (HG + vit. K, 50 µM). Mannitol (20 mM) was used as an osmotic control. Media were maintained at 37°C in a 5% CO₂ atmosphere. After 24 h, segments were washed in PBS and frozen at −80°C for <1 week before use. Graphs show the data from three experiments and results are expressed as a percentage vs. respective NG group.

2.11 Statistical analysis

Results are expressed as mean ± SEM. Statistical comparisons were made between control and diabetic groups using an unpaired t-test. For ex vivo experiments, data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc analysis. A linear regression analysis was performed to detect the relation between selected variables and Pearson’s correlation coefficients r were used to quantify this relation. Statistical significance was set at P < 0.05. The required sample size to detect a minimum of 25% change in the calcium content, imposed as a criterion for the rat model based on previous experiments, was determined a priori with a power analysis with a β = 0.2, α = 0.05, and σ = 0.2, for a n = 13/group.

3. Results

3.1 Animal model of diabetes-associated arterial stiffness

All animals injected with STZ (n = 30) suffered from hyperglycaemia with an average blood sugar level of 21.9 mmol/L (Table 1) and hence were considered diabetic. The increase in body weight was consistent throughout the 9 weeks of the study, although the increase was smaller among diabetic rats, which weighed ~30% less than the age-matched controls at time of sacrifice. Diabetic rats had an increased LV mass index (21% increase vs. controls; Table 1). Nine weeks of diabetes induced an augmentation of arterial stiffness, as demonstrated by the increase in PWV (44% increase vs. controls; Table 1), whereas no change in PP or mean arterial pressure was observed. The aortic calcium content was significantly elevated in diabetic rats compared with controls, and to a greater extent in femoral arteries (respectively 26 and 56% increase vs. controls; Figure 1A and B). The proportion of collagen in sections of the thoracic aorta was higher in diabetic rats (32% increase vs. controls; Figure 1C), and was observed mainly around the elastic lamellae of the vessels.

3.2 γ-Carboxylation of MGP

The amount of cMGP (active) was significantly reduced in the aortas of diabetic rats compared with the control group (36% reduction vs. controls; Figure 2A). Qualitative evaluation of ucMGP (inactive) showed that it was detectable in aortic sections of diabetic rats, although not as an homogenous deposition. ucMGP was predominantly expressed on the elastic lamellae of the vessels (Figure 2B), as well as in the media enclosed in between the stained lamellae. No staining was observed in the aortas of control rats.

3.3 Aortic expression of γ-carboxylase

Diabetic rats had a marked reduction in γ-carboxylase aortic expression (58% reduction vs. controls; Figure 3A). This effect was

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of physiological, metabolical, and haemodynamical parameters between control and diabetic rats</th>
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<tbody>
<tr>
<td></td>
<td>CTRL</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>525.9 ± 9.3</td>
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<tr>
<td>Glucose (mmol/L)</td>
<td>5.33 ± 0.14</td>
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<tr>
<td>Mean arterial pressure (mmHg)</td>
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<tr>
<td>Pulse wave velocity (cm/s)</td>
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<tr>
<td>Pulse pressure (mmHg)</td>
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<tr>
<td>Heart rate (b.p.m.)</td>
<td>368.4 ± 14.9</td>
</tr>
<tr>
<td>Left ventricular mass index (mg/g)</td>
<td>1.77 ± 0.02</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error of the mean; CTRL refers to control group; STZ refers to streptozotocin-treated group.

*p < 0.05 vs. control group.

**p < 0.0001 vs. control group.
significantly correlated to the decrease in cMGP expression (Figure 3B). In addition, an inverse correlation was found between the expression level of γ-carboxylase and the amount of calcification in the aorta (Figure 3C).

### 3.4 Aortic expression and activity of VKOR

The expression of VKOR was unchanged following 9 weeks of diabetes (Figure 4A). Moreover, VKOR activity in aortic microsomes was not modified in diabetic rats compared with controls (Figure 4B).

### 3.5 Vitamin K plasma concentrations

In diabetic rats, plasma concentrations of vitamin K were higher than in control rats (191% increase vs. controls; Figure 5A).

### 3.6 Osteocalcin plasma concentrations

Plasma concentrations of ucOC were similar between control and diabetic rats, whereas the concentrations of γ-cOC were significantly reduced in diabetic rats (13% reduction vs. controls; Figure 5B) and...
thus, the ratio of ucOC/cOC was increased in diabetic rats (24% increase vs. controls; Figure 5C).

3.7 Ex vivo model of hyperglycaemia

In aortic segments cultured in high-glucose medium for 24 h, cMGP was reduced by 17% although this change was not significant (Figure 6A).

Supplementation with vitamin K did not modify cMGP expression. On the other hand, γ-carboxylase expression was significantly reduced (26% reduction vs. controls; Figure 6B), an effect that was prevented by the addition of vitamin K to the culture medium. No significant changes were observed in VKOR expression (Figure 6C).

4. Discussion

The first challenge of this study was to develop a rat model of accelerated arterial stiffness in the context of TD1M in order to investigate the modulation of proteins of interest. Our diabetic model was successful at meeting the primary endpoints, namely elevated aortic PWV and increased MAC in the aorta and femoral arteries, and therefore was considered an appropriate tool to study our hypothesis.

A correlation between PWV and aortic calcium content has previously been demonstrated in the warfarin-vitamin K (WVK) rat model of MAC.30 Thus, the elevated aortic PWV observed in our model is expected to be a result of the increased calcification detected in the aortic media. However, the amount of calcium was lower than in most models of MAC, suggesting that the model represents an early time-point in the development of diabetes-induced MAC. Indeed, MAC is known to be related to the duration of diabetes.18,29,31 Calcification was higher in femoral arteries, in accordance with previous data in diabetic models.29 The increase in aortic collagen in diabetic rats could also have contributed to the elevated PWV, since arterial stiffness depends on the relative contribution of elastin and collagen,32 but this change is expected to be independent of the modulation of MGP: in atherosclerotic lesions, MGP is systematically detected at sites of calcification, but not in areas of fibrous tissue.33 The absence of change in PP was not unexpected because as opposed to PWV depending solely on the stiffness of the vessel, PP varies according to factors other than arterial stiffness; it depends on ventricular ejection, stroke volume, the amplitude of the reflected wave, and the site of reflection points.5,34,35 These parameters were not evaluated in the present study, it is therefore difficult to interpret this result adequately. Nonetheless, STZ-treated diabetic rats had a slower heart rate, higher LV mass index, hypertrophied cardiomyocytes relative to LV mass (Figure S1, Supplementary material online) and are reported to suffer from myocardial diastolic dysfunction as early as after 5 weeks of diabetes.36 These cardiac alterations might have thereby influenced PP.

The amount of cMGP was decreased in the aorta of diabetic rats. To the best of our knowledge, this is the first time that a lack of cMGP in the aorta early in the development of diabetes-induced MAC is demonstrated. This finding supports the hypothesis that the γ-carboxylation of MGP is altered in diabetes, reducing the ability of MGP to inhibit vascular calcification and thereby contributing to the acceleration of arterial stiffness. Whereas previous evidence showed that total MGP mRNA was lower in peripheral arteries of diabetic patients suffering from MAC,21 the present data provide new insights into the amount of functionally active MGP, i.e. capable of binding Ca²⁺, in the diabetic vessel wall. Also, it was shown that ucMGP accumulates at established sites of calcification lesions.24 In our model, calcification lesions were not overtly apparent microscopically. However, ucMGP was present on the elastic lamellae of aortic sections of diabetic rats, suggesting that even before calcification can be detected histologically, MGP γ-carboxylation is impaired at potential sites of hydroxypatite nucleation. Nevertheless,
it is important to emphasize the fact that more abundant inactive MGP (ucMGP) does not cause calcification, but it is rather the lack of functionally active MGP (cMGP) that is expected to contribute to the development of MAC. The specific role of insulin in the regulation of MGP expression is currently unclear, with conflicting results in rat and mouse models of hyperinsulinaemia or following direct stimulation of vascular smooth muscle cells from Wistar-Kyoto rat.

A major finding of this study is the marked reduction in the expression of γ-carboxylase in the aorta of diabetic rats. We propose that this low expression of γ-carboxylase results in altered γ-carboxylation of MGP, a prerequisite for its activity as an inhibitor of calcification. In turn, this deficit in active MGP leads to a disequilibrium between inhibitors of calcification and osteogenic signals, promoting an extracellular environment prone to pathological mineralization in diabetes. The fact that the γ-carboxylase protein level was positively correlated to cMGP abundance, and negatively correlated to the aortic calcium content reinforces this rationale. Impaired γ-carboxylation is observed in other pathologies such as urolithiasis, in which the formation of calcium oxalate calculus has been attributed to a decreased expression of γ-carboxylase in renal cortex specimens and a reduced γ-carboxylation of the VKD protein urinary prothrombin fragment-1, a potent inhibitor of calcium oxalate crystal growth. Likewise, the PXE-like syndrome is a disorder characterized by the mineralization of elastic fibres due to a mutation in the GGCX gene coding for γ-carboxylase. This leads to a deficit in γ-carboxylation of VKD proteins such as MGP and OC, a pro-osteoblastic protein synthesized in bone, as evidenced by a higher ratio of undercarboxylated/γ-carboxylated (uc/c) of both MGP and OC in serum. Similarly, our results show that diabetic rats also had a higher plasma ratio of ucOC/cOC, adding further evidence to support the hypothesis of a γ-carboxylation disorder in T1DM.

It has been suggested that the accumulation of inactive ucMGP in areas of calcification in femoral arteries of diabetic patients could be a consequence of local vitamin K deficiency. Given that VKOR is responsible for providing the necessary reduced vitamin K to γ-carboxylase in order to activate MGP, we speculated that decreased aortic cMGP and therefore increased MAC in diabetes could be attributable to a defect in VKOR. However, neither VKOR expression nor activity was modified in the aorta of diabetic rats, suggesting that if a vitamin K deficiency in the vasculature is indeed present, it would not be related to VKOR’s ability to reduce vitamin K.

An increased ucOC/cOC ratio is frequently used as a functional biomarker to indicate a low vitamin K status. Surprisingly, elevated plasma concentrations of vitamin K were found in diabetic rats compared with controls despite a higher ratio of ucOC/cOC. This finding strengthens the hypothesis that γ-carboxylase is, at least in part, responsible for the γ-carboxylation deficit in vivo in diabetic rats. The elevated circulating levels of vitamin K could be explained by the impairment in lipid metabolism in T1DM, notably in terms of altered clearance of chylomicron remnants (CRs), thus promoting vitamin K retention in plasma given that CRs are the main carriers of vitamin K in the blood. This situation is observed in individuals carrying the apolipoprotein E genotype E2/2 in which CRs clearance is thought to be the slowest, causing vitamin K to remain in the circulation for a prolonged period of time and to attain higher concentrations. Furthermore, plasma levels of vitamin K correlate positively with triglycerides concentrations and STZ-treated rats are indeed reported to suffer from hypertriglyceridaemia as a result of a defect in lipoprotein removal from the plasma. In our study, a high glucose concentration per se reduced γ-carboxylase expression in aortic segments cultured ex vivo, once again highlighting the role of γ-carboxylase as a potential culprit for MAC in diabetes. In these isolated aortic rings taken from healthy rats, vitamin K supplementation prevented the glucose-induced reduction of γ-carboxylase expression, emphasizing the fact that lipid metabolism alterations as well as other structural and functional changes in the vasculature might alter proper vitamin K uptake by target cells in diabetic animals and possibly result in a local deficiency even when elevated circulating levels of vitamin K are present.
The extremely small amount of microsomes in vascular tissue represented a limitation to this study and required to pool a large number of aortas in order to obtain the necessary material for enzymatic assays. A total of 60 rats allowed for only one assay, and it was chosen to be VKOR to rule out its potential contribution to the reduction in aortic cMGP, as the expression of VKOR was not altered. γ-Carboxylase activity was therefore not measured, but the marked reduction in its expression was considered to be a strong evidence.

In conclusion, our findings suggest that a reduction in active cMGP, through an impaired expression of γ-carboxylase, is involved in the early development of MAC in diabetes and therefore, in the acceleration of arterial stiffness. Moreover, a defect in vitamin K uptake by target cells could also be involved in diabetes-associated MAC. Further studies are underway to elucidate the signalling pathways implicated in γ-carboxylase regulation in diabetes.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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