Effects of Atorvastatin and Insulin in Vascular Dysfunction Associated With Type 2 Diabetes

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Summary
Atorvastatin and insulin have distinct mechanisms of action to improve endothelial function. Therefore, we hypothesized that atorvastatin and insulin therapies alone or in combination could have beneficial effects on endothelium-dependent vascular reactivity, oxidative stress, inflammation and metabolic parameters in Goto-Kakizaki (GK) rats, a model of type 2 diabetes fed with atherogenic diet (GKAD). In parallel with the development of diabetes and lipid profile, the generation of oxidative stress was determined by measurement of lipid peroxides and oxidized proteins and the presence of inflammation was evaluated by assessing C-reactive protein (CRP). Additionally, endothelial dependent and independent vascular sensitivity to acetylcholine and sodium nitroprusside were evaluated. GKAD showed increased carbonyl stress, inflammation, fasting glycemia, dyslipidemia and endothelial dysfunction when compared to control GK rats. Noteworthy, supplementation with insulin deteriorated endothelial dysfunction while atorvastatin induced an improvement. Atorvastatin and insulin therapies in combination improved metabolic parameters, CRP levels and insulin resistance indexes and ameliorated endothelial dysfunction in GKAD rats while they were unable to reduce urinary 8-isoprostranes and plasma carbonyl compounds. The therapeutic association of atorvastatin and insulin provided a better metabolic control with a reduction in endothelial dysfunction in GKAD rats by a mechanism that involves an improvement in systemic inflammation.

Key words
Endothelial dysfunction • Type 2 diabetes • Insulin • Atorvastatin • Goto-Kakizaki rats

Introduction
3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are more than just cholesterol lowering drugs (Koh et al. 2011). The effects of statins in the cardiovascular system include, among others, the improvement of endothelium-dependent relaxation through a mechanism that involves endothelial nitric oxide synthase up-regulation, a reduction in the progression of arteriosclerosis, an antihypertensive effect and beneficial actions on cardiac function (Takemoto and Liao 2001, Ii and Losordo 2007).

Insulin plays an important role in maintaining both hemodynamic and metabolic homeostasis under healthy conditions. Insulin has important vascular actions to stimulate production of nitric oxide (NO) from endothelium. This leads to capillary recruitment, vasodilation, increased blood flow, and subsequent augmentation of glucose disposal in classical insulin target tissues. Thus, insulin treatment not only reduces glycemia but may also directly increase NO production through 1-phosphotidylinositol 3-kinase (PI-3K) signalling (Muniyappa et al. 2007). In insulin-resistant states, the selective impairment in the PI-3K/Akt/endothelial nitric oxide synthase (eNOS) pathway and the augmented ERK1/2 signaling cascade in vascular endothelium lead to decreased NO availability.
and enhanced endothelin-1 (ET-1) production, thereby tilting the balance between the vasodilator and vasoconstrictor actions of insulin toward endothelial dysfunction and hypertension (Huang 2009).

Atorvastatin and insulin have distinct mechanisms of action to improve endothelial function. Therefore, we hypothesized that combination therapy has additive beneficial effects to simultaneously improve endothelial dysfunction, inflammation and oxidative stress profiles in GK rats fed with an atherogenic type of diet.

Methods

Experimental animals

Goto-Kakizaki rats were obtained from our local breeding colony (Animal Research Center Laboratory, University Hospital, Coimbra, Portugal). Rats were divided in 5 experimental groups accordingly [GK diabetic rats fed with a normal diet (GK control), GK diabetic rats fed with an atherogenic diet (AD, GKAD), and GK rats fed an AD diet treated with atorvastatin (GKAD+A), insulin (GKAD+I) or the association of atorvastatin and insulin (GKAD+A+I)]. Special high fat diet (AD) was obtained from Safe (France) and contained 70% AO4 standard chow diet, 7.5% cocoa butter, and 1.25% cholesterol. The animals were maintained with AD diet between 2 and 6 months of age. All the animals were used with 6 months old.

All animals received care in accordance with the Portuguese Law on Experimentation with Laboratory Animals (last amendment, 2004), which is based on the principles of laboratory animal care as adopted by the EC Directive 86/609/EEC for animal experiments.

Treatment administration

Insulin and atorvastatin were administered during one month (between 5 and 6 month old) before sacrifice. Insulin (Mixtard 30 Novolet, from Novo Nordisk A/S, Denmark) was administered twice a day subcutaneously according to glucose levels: glycemia between 150 and 199 mg/dl, 2U of insulin; glycemia between 200 and 299 mg/dl, 4U of insulin; glycemia higher than 300 mg/dl, 6U of insulin. Atorvastatin (Pfizer, USA) was administered orally (10 mg/kg/day) diluted in water.

Determination of metabolic and oxidative stress parameters

After a 15 h fast, animals were anesthetized with ketamine/chlorpromazine [ketamine chloride (75 mg/kg, im, Parke-Davis, Ann Arbor, MI, USA) and chlorpromazine chloride (2.65 mg/kg, im, Lab. Vitória, Portugal)] and killed by decapitation. Blood was taken by heart puncture for determination of lipids, carbonyl compounds and insulin. Fasting plasma lipids (total and HDL cholesterol and triglycerides, Olympus-Diagnóstica Portugal, Produtos de Diagnóstico SA, Portugal) and plasma insulin levels were quantified using commercially available kits and by an in-house enzyme-linked immunosorbent assay, respectively. Plasma free fatty acids (FFA) levels were evaluated using enzymatic assay kits (Roche Applied Science, Portugal). Rats were placed in metabolic cages for 24 h and urine collected. Urinary 8-isoprostanates (OXIS health Products, Portland, OR, USA), C reactive protein (CRP; BD Biosciences Pharmingen, CA, USA) and plasma carbonyl protein concentrations (Cayman Chemical Company, USA) were measured using ELISA kits.

For glucose tolerance tests, rats were fasted overnight and were given an intraperitoneal injection of glucose (1.75 g kg\(^{-1}\) body weight). Blood glucose was determined by sampling from the tail vein at 0, and 120 min after injection by a glucose-oxidase method using a glucometer (Glucometer-Elite-Bayer, Portugal S.A.) and compatible reactive test strips. To assess insulin resistance in the fasted state, the homeostasis model assessment of insulin resistance (HOMA) and quantitative insulin-sensitivity check index (QUICKI), were calculated, as previously described (Sena et al. 2008). HOMA was calculated as \( [(\text{G}_0) \times (\text{I}_0)/22.5] \) where \( \text{G}_0 \) is the fasting glucose levels (mmol/l) and \( \text{I}_0 \) is the fasting insulin levels (µU/ml). QUICKI was calculated as \( 1/[(\log(\text{G}_0)+\log(\text{I}_0))] \), where \( \text{G}_0 \) is fasting glycemia (mg/dl) and \( \text{I}_0 \) is fasting insulin levels (µU/ml). Index of atherogenicity was calculated as total cholesterol/HDL-cholesterol.

Aortic nitrite levels

Nitrite levels were determined as an index of NO generation in aortic homogenates by the Griess reaction after conversion of nitrate to nitrite by nitrate dehydrogenase as previously described (Majithiya et al. 2005). An aliquot of the supernatant was mixed with an equal volume of Griess reagent (Sulfanilamide 1 % w/v; naphthylethenediamine dihydrochloride, 0.1 % w/v; and orthophosphoric acid, 25 % v/v) and incubated at room temperature for 10 min. The absorbance of the samples at 540 nm was determined and compared with those of known concentrations of sodium nitrite. The amount of
nitrite formed was normalized to the protein content of the respective aorta.

**Isometric tension studies**

Aorta were rapidly excised and freed of connective tissue. The aorta was divided into two segments (4-mm width). Ring segments were mounted between stainless steel triangles into individual organ chambers filled with oxygenated (95% O2, 5% CO2) modified Krebs-Henseleit buffer (37 °C, pH 7.4) (composition in mM: NaCl 119, KCl 4.7, CaCl2 1.6, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, Glucose 11.0). Indomethacin in a concentration of 10 µM was present in the experiments to inhibit prostaglandin synthesis. Aortic rings were subject to a resting tension of 14.7 mN. After equilibration for 60 min all vessels were preconstricted with 0.3 µM phenylephrine. Ligand stimulated receptor-mediated NO bioavailability was assessed by a concentration-dependent relaxation to acetylcholine (ACh, 10⁻⁹ to 10⁻² M), whereas sodium nitroprusside (SNP, 10⁻⁹ to 10⁻³ M) was used as an endothelium-independent agonist. Relaxation responses to ACh and SNP were expressed as percentage of relaxation from a submaximal phenylephrine-induced constriction and dose-response curves were obtained as previously described (Sena et al. 2008, 2011).

**Statistical analysis**

All data were analyzed by standard computer programs (GraphPad Prism PC Software version 3.0, ANOVA) and are expressed as mean ± SEM (n=16 individual animals per group). Significant differences were evaluated using either the t-test or ANOVA. P<0.05 was considered significant. Dose response curves were fitted by nonlinear regression with simplex algorithm. Relaxation responses were given as the percentage of phenylephrine-preconstriction. Comparisons of dose-response curves were evaluated by 2-way ANOVA for repeated measures.

**Results**

Atherogenic diet resulted in an increase in body weight and fasting glycemia, while no changes were observed in insulin resistance, when compared with GK control rats. Food consumption was similar in all the experimental groups (Table 1). Treatment with atorvastatin significantly reduced fasting blood glucose and ameliorated insulin resistance indexes. Treatment with insulin had similar effects. The therapeutic combination of atorvastatin and insulin significantly reduced fasting and 2 h glycemia and further improved insulin resistance indexes when compared with GKAD rats (Table 1).

GKAD rats exhibited higher levels of triglycerides, total-cholesterol and FFAs when compared to diabetic control rats, as well as an elevated atherogenic index, while HDL-cholesterol levels were significantly lower than those in GK control rats. Atorvastatin and insulin treatments significantly reduced these indexes (Table 1).

**Table 1. Influence of atorvastatin (GKAD+A), insulin (GKAD+I) and the association of both (GKAD+A+I) on body weight, blood glucose (fasting glucose – FBG and 2 h glycemia), insulin levels and insulin resistance indexes (HOMA and QUICKI) in atherogenic diet fed GK (GKAD) rats (mean ± SEM of 8-16 animals).**

<table>
<thead>
<tr>
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<th>GK</th>
<th>GKAD</th>
<th>GKAD+A</th>
<th>GKAD+I</th>
<th>GKAD+A+I</th>
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<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>345.2±8.4</td>
<td>387.3±4.3§§§</td>
<td>400.9±7.0§§§</td>
<td>391.7±5.0§§§</td>
<td>390.8±5.1§§§</td>
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<tr>
<td><strong>Diet (g/rat/day)</strong></td>
<td>23±0</td>
<td>19±2</td>
<td>20±1</td>
<td>19±1</td>
<td>18±1</td>
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<tr>
<td><strong>FBG (mg/dl)</strong></td>
<td>93.1±2.5</td>
<td>140.2±2.8§§§</td>
<td>118.2±2.3***</td>
<td>97.3±2.1***</td>
<td>91.0±1.2***</td>
</tr>
<tr>
<td><strong>Glycemia at 2 h (mg/dl)</strong></td>
<td>299.4±7.2</td>
<td>303.2±14.9</td>
<td>288.3±10.1</td>
<td>268.5±13.7</td>
<td>222.5±11.9***</td>
</tr>
<tr>
<td><strong>Insulin (µU/ml)</strong></td>
<td>37.0±3.4</td>
<td>32.8±5.6</td>
<td>25.1±3.0</td>
<td>23.9±4.3</td>
<td>21.7±5.3</td>
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<tr>
<td><strong>HOMA</strong></td>
<td>11.2±1.3</td>
<td>14.7±2.5</td>
<td>7.3±0.8§</td>
<td>5.3±1.0§§</td>
<td>4.8±1.2§§</td>
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<tr>
<td><strong>QUICKI</strong></td>
<td>0.286±0.01</td>
<td>0.271±0.01</td>
<td>0.292±0.01§</td>
<td>0.309±0.01***</td>
<td>0.320±0.01***</td>
</tr>
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§§§ P<0.001 vs GK rats; φ P<0.05, φφ P<0.01, φφφ P<0.001 vs GKAD rats.
lower. The levels of total cholesterol and triglycerides remained high and did not change in the different GKAD groups while HDL-cholesterol levels significantly increased with all therapeutic approaches (Table 2). Additionally, all GKAD treated groups showed a decrement in FFAs levels. Moreover, atorvastatin and insulin in combination was the only treatment able to significantly reduce atherogenic index (Table 2).

Atherogenic diet significantly increased carbonyl groups and CRP levels while a decrement in aortic nitrite/nitrate levels occurred. The different therapeutic approaches failed to diminish urinary 8-isoprostanes and plasma carbonyl compounds. Atorvastatin in association with insulin, significantly increased aortic nitrite/nitrate levels. CRP levels were not significantly different between GKAD rats and the groups treated with atorvastatin or insulin while the therapeutic combination of atorvastatin and insulin significantly decreased these levels (Table 3).

As it was found in our previous experiments, 6 months old GK rats endothelium-mediated vascular relaxation of phenylephrine-precontracted aorta arterial rings in response to ACh was impaired compared with age-matched Wistar rats, but the endothelium-independent relaxations to SNP were similar in both strains (Sena et al. 2011). Atherogenic diet significantly impaired vascular relaxation in response to ACh in GK rats. Maximal endothelium-mediated relaxation of phenylephrine-precontracted rings in response to ACh declined by 15 % when compared with control GK rats (Fig. 1). Noteworthy treatment with insulin further impaired endothelial dysfunction and supplementation

### Table 2. Effects of atorvastatin (GKAD+A), insulin (GKAD+I) and the association of both (GKAD+A+I) on lipid profile (triglycerides, total and HDL-cholesterol and free fatty acids, FFAs) and atherogenic index in atherogenic diet fed GK (GKAD) rats (mean ± SEM of 8-16 animals).

<table>
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<tr>
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<th>GK</th>
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<th>GKAD+A+I</th>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>149.5±11.6</td>
<td>222.9±20.0⁷⁷</td>
<td>271.7±23</td>
<td>193.7±8.5</td>
<td>196.4±12.2</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>88.9±1.9</td>
<td>100.4±5.3⁷</td>
<td>116.4±8.1</td>
<td>105.1±3.9</td>
<td>96.5±4.3</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>61.4±1.3</td>
<td>48.8±0.9⁷⁷</td>
<td>59.4±2.6⁹⁹</td>
<td>53.7±1.0⁹⁹</td>
<td>55.3±1.7⁹⁹</td>
</tr>
<tr>
<td>Atherogenic index</td>
<td>1.45±0.01</td>
<td>2.02±0.37⁷⁷</td>
<td>1.87±0.05</td>
<td>1.96±0.06</td>
<td>1.74±0.04⁹</td>
</tr>
<tr>
<td>Free fatty acids (mM)</td>
<td>0.79±0.04</td>
<td>1.13±0.04⁷⁷</td>
<td>0.81±0.07⁹⁹⁹</td>
<td>0.79±0.06⁹⁹⁹</td>
<td>0.69±0.09⁹⁹⁹</td>
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§ P<0.05, §§§ P<0.001 vs GK rats; φ P<0.05, φφ P<0.01, φφφ P<0.001 vs GKAD rats.

### Table 3. Effects of atorvastatin (GKAD+A), insulin (GKAD+I) and the association of both (GKAD+A+I) on oxidative stress parameters (8-isoprostanes and carbonyl groups), aortic nitrite/nitrate levels and CRP in atherogenic diet fed GK (GKAD) rats (mean ± SEM of 8-16 animals).

<table>
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<tr>
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<th>GK</th>
<th>GKAD</th>
<th>GKAD+A</th>
<th>GKAD+I</th>
<th>GKAD+A+I</th>
</tr>
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<tbody>
<tr>
<td>8-Isoprostane (ng/24h)</td>
<td>23.1±2.26</td>
<td>19.6±1.1</td>
<td>25.5±1.2⁶</td>
<td>21.8±2.7</td>
<td>20.5±2.1</td>
</tr>
<tr>
<td>Carbonyl groups (mmol/ml)</td>
<td>23.5±1.1</td>
<td>38.1±3.0⁷⁷</td>
<td>31.0±2.2</td>
<td>35.8±3.7</td>
<td>33.8±2.7</td>
</tr>
<tr>
<td>Nitrite/nitrate levels (pmol/mg dry wt aorta)</td>
<td>280.3±11.9</td>
<td>232.4±14.9⁶</td>
<td>263.0±6.4</td>
<td>218.7±12.6⁷⁷</td>
<td>285.6±4.9⁶</td>
</tr>
<tr>
<td>CRP (μg/ml)</td>
<td>34.4±1.2</td>
<td>43.3±3.2⁶</td>
<td>35.4±3.4</td>
<td>41.0±5.4</td>
<td>31.0±3.7⁹</td>
</tr>
</tbody>
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§ P<0.05, §§§ P<0.001 vs GK rats; φ P<0.05 vs GKAD rats; φφ P<0.01 vs GKAD rats.
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with atorvastatin and atorvastatin + insulin improved endothelium-dependent relaxation in response to ACh (Fig. 1). No differences on maximal relaxation were observed in the dose-response curves for SNP between the different groups (Fig. 2). Vascular sensitivity to SNP was decreased in GKAD rats (Fig. 2). Indeed, pD2 values of GKAD were significantly decreased when compared with age-matched controls (5.42±0.03 in GKAD vs 6.43±0.04 in GK control rats, p<0.001). Atorvastatin alone or in association with insulin significantly improved endothelium-independent vascular relaxation (Fig. 2) with an increment in pD2 value (GKAD+A 5.77±0.096, p<0.001 vs GKAD; GKAD+A+I 6.2±0.04, p<0.001 vs GKAD). These results indicated that treatment with atorvastatin and insulin in combination was able to improve endothelial dysfunction in GKAD rats.

Discussion

Endothelial dysfunction is a hallmark of diabetes and insulin-resistant states and is characterized by reduced effective vascular NO action (McFarlane et al. 2002). An appropriate balance between NO, a vasodilator, and ET-1, a vasoconstrictor, is struck when the endothelium is functioning normally (von Haehling et al. 2003). Endothelial dysfunction may manifest as an imbalance between vasodilation and vasoconstriction (Sena et al. 2013).

In this work we show that atherogenic diet induced an increment in carbonyl stress, inflammation, fasting glycemia, lipid profile, atherogenic index and endothelial dysfunction when compared with control GK rats. In accordance, it is well known that atherogenic diets promote dyslipidemia, insulin resistance and endothelial dysfunction in several rodent models (Fernández-Real and Ricart 2003).

In this study we show that atorvastatin treatment was beneficial on glucose metabolism (fasting glucose and 2 h glucose). When compared with control GK rats, atorvastatin treatment resulted in an improvement of fasting and 2 h glucose (ip glucose tolerance test). This observation is consistent with previous studies that showed that statin therapy can improve the parameters of glucose metabolism in diabetic and nondiabetic patients (Paniagua et al. 2002, Costa et al. 2003, Sonmez et al. 2003, Watts et al. 2003, Güclü et al. 2004). High-dose statin therapy, however, deteriorates glycemic control in patients with diabetes (Simsek et al. 2012). Additionally, monotherapy with atorvastatin was able to decrement FFAs, HOMA and augment HDL-cholesterol and improved endothelial function. An improvement in HOMA index indicates that insulin resistance is ameliorated due to a decrement in fasting glucose.
However, it is unclear whether this improvement was achieved by decreased gluconeogenesis or by increased uptake of glucose in muscle and fat, or both. Previous studies indicated that the flux and turnover of portal free-fatty acids is crucial for the development of insulin resistance (Kabir et al. 2005). Indeed, we observe an inhibition of FFAs levels after treatment with atorvastatin in addition to the decrement in insulin resistance index (HOMA).

Treatment with 10 mg/kg atorvastatin in our experimental conditions did not affect the levels of total cholesterol and triglycerides compared to control GK group. Importantly, HDL-cholesterol levels significantly increased while total-cholesterol remained unchanged. Similar results were obtained by others (Hayashi et al. 2005, Nagotani et al. 2005, Tanaka et al. 2007) with identical doses of atorvastatin in other animal models. Other studies have shown large discrepancies in the effectiveness of HMG-CoA inhibitor in lowering plasma cholesterol levels among animal species (Endo et al. 1979).

Treatment with insulin as monotherapy was also able to decrement fasting glucose and FFAs and to improve insulin resistance indexes and augment HDL-cholesterol levels. Importantly, these metabolic effects were not effective to promote beneficial outcomes at the endothelium level as in the atorvastatin treated groups. All therapies failed to reduce the oxidative stress parameters evaluated. Insulin treated groups received human insulin according to their glycemic levels; as in humans. Importantly, there were no episodes of hypoglycemia, under our conditions, and normoglycemia was not achieved. Additionally, insulin treatment had no effect on daily food intake and on levels of triglycerides. This corroborates the lack of anabolic effect of insulin observed in our experimental conditions. In agreement with our study, Suzuki and co-workers (2007) have treated GK rats (for 5 weeks) without any effects on body weight.

Herein, we have shown that supplementation with atorvastatin and insulin in combination decreased blood glucose, FFAs, atherogenic index, CRP and improved insulin resistance indexes, aortic nitrite/nitrate levels and endothelial dysfunction in GKAD rats.

In agreement with our studies, it was previously described that statins ameliorate the abnormal vascular relaxation and partially restore NO production in the aorta of diabetic mice (Lefer et al. 2001). Previously studies have suggested that statins may help to correct the imbalance between vasodilation and vasoconstriction by enhancing the activity of eNOS and thus increasing the production of NO (Laufs et al. 1998). They may also reduce the synthesis of ET-1 (Hernandez-Perera et al. 1998, Puddu et al. 2001). Furthermore, it was previously shown that after 4 weeks of therapy with simvastatin there was an increment in the NO-mediated vasodilation. This effect appeared to be independent of simvastatin’s lipid-lowering effects (O’Driscoll et al. 2007). It was previously described that statins increase the expression of eNOS (Laufs and Liao 1998), but also enhance eNOS activity by decreasing caveolin abundance (Feron et al. 2001) and by activation of the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway (Kureishi et al. 2000). Statins have also been shown to increase GTP cyclohydrolase1 mRNA expression in endothelial cells and to elevate intracellular tetrahydrobiopterin (BH4) levels (Hattori et al. 2003).

Other authors suggested that atorvastatin can exert anti-oxidant, anti-inflammatory benefits (Wagner et al. 2000, Mason et al. 2004) an effect that was not seen in our experimental conditions. Previous studies have shown that different statins are able to decrement CRP levels in both animal models and humans (Devaraj et al. 2011). New pleiotropic effects have been suggested for statins. The Justification for the Use of statins in Primary prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) trial showed that individuals without overt cardiovascular disease, with a LDL cholesterol below the current threshold for treatment, an elevated CRP would benefit from statin therapy. Statin therapy can reduce both LDL cholesterol and hsCRP, in subjects with metabolic syndrome (Devaraj et al. 2011). In addition to the anti-inflammatory properties, the capacity of statins to alter circulating levels of several adipokines known to affect glucose homeostasis, including adiponectin, leptin, visfatin and resistin, may beneficially alter glycemic status (Kostapanos et al. 2010, Buldak et al. 2012).

Statins have been described to improve flow-mediated dilation in humans (Morrow et al. 2012). Atorvastatin has been shown to normalize endothelial function and reduces oxidative stress by inhibiting vascular NADPH oxidases and preventing eNOS uncoupling by an up-regulation of GTP cyclohydrolase1 (Wenzel et al. 2008). Although atorvastatin, in our experimental conditions, did not normalize endothelial function or had anti-oxidant and anti-inflammatory properties, as previously described by others (Nissen et al. 2006, Patel et al. 2007), the animal model and the
therapeutic (time and concentration) was considerable different and can explain the discrepancies. Importantly, atorvastatin significantly improved endothelium-dependent and independent vascular relaxation probably due to its metabolic effects. Noteworthy, supplementation with insulin impaired ACh-induced aortic relaxation, deteriorating the endothelial dysfunction observed in GKAD rats. Insulin is an essential hormone of metabolic homeostasis with a vasodilator action through PI3K/AKT pathway-dependent eNOS activation (Muniyappa et al. 2007). Insulin can also modulate eNOS activity by increasing BH4 synthesis (Shinozaki et al. 1999, Huang 2009). It has been previously described that insulin can activate the sympathetic nervous system and also stimulate the secretion of the vasoconstrictor ET-1 from vascular endothelium (Muniyappa et al. 2007), these effects can explain the aggravation of endothelial dysfunction. It was previously described that in aortas from rats with established STZ-induced diabetes, insulin leads to an enhanced aortic peroxynitrite generation and that this increment causes a dysfunction of endothelium dependent relaxation, an impairment of sarcoplasmic reticulum Ca2+ ATPases (SERCA) function. Further, it was found that addition of a peroxynitrite scavenger normalized this impaired relaxation (Kobayashi et al. 2007). These are important findings concerning the action of insulin in diabetic arteries, and in agreement with our results suggest that an insulin-induced production of peroxynitrite may be involved in mediating diabetic complications.

Furthermore, in this work we also show that atorvastatin in combination with insulin improves endothelial function to a greater extent than monotherapy with atorvastatin and significantly decreases atherogenic index and CRP levels in GK rats fed with an atherogenic diet. Noteworthy, monotherapy with atorvastatin was able to reduce fasting glucose, FFAs and improved insulin resistance indexes and endothelial function. All therapeutic approaches failed to reduce oxidative stress in Goto-Kakizaki rats fed with an atherogenic diet. Thus, the therapeutic association of atorvastatin and insulin ameliorated endothelial dependent vasodilation in GKAD rats by a mechanism that is likely due to an increment in aortic NO bioavailability and a decrement in systemic inflammation.

In conclusion, atherogenic diet induced an increment in carbonyl stress, inflammation, fasting glycemia, lipid profile and endothelial dysfunction when compared with control GK rats. Noteworthy, supplementation with insulin deteriorated endothelial dysfunction while atorvastatin induced an improvement. Atorvastatin and insulin therapies in combination decreased CRP, atherogenic index, FFAs and improved endothelial dysfunction and insulin resistance indexes in GKAD rats. The therapeutic association of atorvastatin and insulin provided a better metabolic control with a reduction in endothelial dysfunction in GKAD rats by a mechanism that involves an increment in NO bioavailability an improvement in systemic inflammation.

Conflict of Interest
There is no conflict of interest.

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