Abstract
Objective: The aim of the present study was to evaluate the indicative role of hydrogen peroxide \((H_2O_2)\) in oxidative stress.

Methods: 200 patients with type 2 diabetes were enrolled and 200 matched controls. \(H_2O_2\), glycated hemoglobin \((HbA1c)\), free fatty acids \((FFA)\) and homocysteine were determined by commercial kits. Quantitative insulin sensitivity check index \((QUIKI)\) was calculated.

Results: \(H_2O_2\) concentration was increased fourfold in type 2 diabetes patients compared to controls. Several correlations were found: between \(H_2O_2\) and \(HbA1c\) \((r = 0.85, p < 10^{-3})\), between \(H_2O_2\) and \(FFA\) \((r = 0.9, p < 10^{-3})\), between \(H_2O_2\) and homocysteine \((r = 0.5, p < 10^{-3})\), between \(H_2O_2\) and \(QUIKI\) \((r = 0.92, p < 10^{-3})\), between the presence of \(H_2O_2\) and arterial hypertension \((t = -4, p < 10^{-3})\) and between arterial hypertension and homocysteine \((t = -7, p < 10^{-3})\).

Conclusions: The overproduction of \(H_2O_2\) generated by hyperglycemia, increased dose of \(FFA\) and hyperhomocysteinemia, amplifies the insulin resistance.

Keywords — \(H_2O_2\), \(HbA1c\), \(FFA\), homocysteine, type-2 diabetes

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I. INTRODUCTION

Systemic glucose homeostasis in type 2 diabetes is a key pathophysiological function. Despite pharmaceutical treatments and dietary measures it is difficult to control blood glucose levels in diabetic patients. Thereby glycemic instability and chronic hyperglycemia promote the development of oxidative stress and generate numerous complications in diabetic patients.

Brownlee proposed an explanation for the pathogenesis of vascular complications of diabetes. Oxidative stress is the important metabolic pathway involved in the development of diabetes. By interaction with other pathways, it is responsible for all complications [1]. Increased glucose levels in endothelial cells induce an excessive substrate production to mitochondria. The overproduction of reactive oxygen species \((ROS)\) via oxidative phosphorylation, induces an oxidative stress and ultimately an endothelial dysfunction.

Distinct mechanisms \(QUIKI\) activated by hyperglycemia, are involved in the generation of ROS, leading to both vascular and kidney damage. The most important mechanisms are the increased polyol and hexosamine pathways, the over activation of the transcription factor NF kappa B, the angiotensin 2 synthesis stimulation, the protein kinase C activation, the overproduction of advanced glycation end products and the excessive NADH, H+ and FADH2 supplying the respiratory chain [2].

In order to demonstrate that oxidative stress is associated with chronic hyperglycemia, several biochemical parameters must be analyzed. The most important marker of oxidative stress establishment remains so far unclear. The aim of the present study was to evaluate the functional role of hydrogen peroxide \((H_2O_2)\) in oxidative stress.

II. METHODS

Study population: After hospital approvals were obtained, 200 confirmed type 2 diabetes patients were ascertained from the Endocrinology Department at CHU Farhat Hached Sousse, with no previous diagnosis of thyroid, adrenal or kidney failure. A total of 200 controls were recruited among blood donors in regional blood transfusion center of Sousse. Patients and controls were matched on age and sex. All participants have signed informed consent. Clinical data and hypoglycemic treatment are presented in Table 1.

Experimental procedures: Two blood samples were collected from each fasted participant. In order to determinate the insulin and homocysteine concentrations, the first sample was carried out on lithium heparin. The second was performed on EDTA to assess glycated hemoglobin \((HbA1c)\), hydrogen peroxide \((H_2O_2)\) and free fatty acids \((FFA)\).

FFA concentration was measured by an enzymatic method \((Randox, Antrim, UK)\). \(HbA1c\) was determined by immunoturbidimetric assay \((Roche Diagnostics, Mannheim, Germany)\) and total hemoglobin by colorimetry. The two concentrations were measured after blood hemolysis collected on anticoagulant; their ratio provided the percentage of \(HbA1c\).
H$_2$O$_2$ was determined by the colorimetric technique (PerOx, Immune diagnostik, Wiesenstr, Bensheim). H$_2$O$_2$ concentration was obtained using a microtiter plate reader Σ 960 (Mettech, Nangang, Taipei, Taiwan) at 450 nm. Homocysteine was determined by an immunological method of fluorescence polarization controller (AXSYM, Abbott, Wiesbaden, Germany). Insulin was measured by an immunoradiometric assay using a dedicated gamma counter (QuikI) according to the formula of Perseghin et al. [3]:

$$\frac{1}{\text{Log (insulin)}} + \frac{\log \text{(glucose)}}{\log \text{(FFA)}}.$$

Sample Extraction and High Performance Liquid Chromatography (HPLC) Analysis: Cells in 10 mL medium were incubated with 25(OH)D$_3$ (100 nM) + $[^3]$H-25(OH)D$_3$ (~200,000 dpm) for 24 hrs. Cells were then mixed with equal volume of acetonitrile. The mixture was vortexed for 30 seconds, and centrifuged at 3000 rpm for 10 min. The supernatant was applied to a SepPak C18 cartridge (Waters Associates, Milford, MA) that was pre-washed with 5 mL hexane, 5 mL chloroform, 5 mL methanol, followed by 5 mL water. After the sample application, the column was washed with 5 mL water, and then eluted with 4 mL of acetonitrile. The sample was dried under nitrogen and resuspended in 100 µL of solvent containing 50% acetonitrile and 50% Mobile Phase A (see below). Sample analysis was performed with an Agilent 1100 HPLC System equipped with a Packard Radimetric 525STR radiodetector using a Phenomenex Luna 5 µm Phenyl-Hexyl column (4.6 × 250 mm) and a Phenomenex Octadeyl guard column (4 × 3 mm). The mobile phase consisted of (A) 95% of 10 mM ammonium acetate and 5% (r = 0.9, p<0.01, Figure 2). As shown in Figure 3, there is a positive correlation between homocysteine concentration and H$_2$O$_2$ concentration (r = 0.5, p<0.01). We also found a negative correlation between QUIKI and H$_2$O$_2$ (r = -0.92, p<0.01, Figure 4). Finally, two additional correlations were found: the first one between arterial hypertension and the concentration of H$_2$O$_2$ (t = -4, p <0.01) and the second one between arterial hypertension and homocysteine (t = -7 p<0.01).

### IV. Discussion

The H$_2$O$_2$ concentration determined was increased fourfold in type 2 diabetes compared with controls. Our results are similar to those found in a study that showed an increased mitochondrial H$_2$O$_2$ production in hyperglycemic mice [4].

The positive correlation found between the HbA1c and the H$_2$O$_2$ concentrations suggest that ROS production is as intensive as glycemia imbalance. Indeed, some studies have shown that glucose metabolism, via pentose pathway, is less than twice in cells with over glucosed medium (33 mM). They concluded that increased H$_2$O$_2$ in diabetics is due to the pentose pathway reduction [5]. Other studies have reported that reaction between AMADORI products such as HbA1c and molecular oxygen may produce superoxide anion (O$_2^-$) or H$_2$O$_2$ [6].

We have identified a positive correlation between FFA concentration and H$_2$O$_2$ concentration (Fig. 2, r = 0.9, p<0.01). The correlation between FFA and H$_2$O$_2$ shows FFA decoupling effect on mitochondria. In one hand this leads to ATP decrease, partially responsible on insulin secretion inhibition verified by low insulin concentration in our diabetic samples. In the other hand FFA decoupling effect increases anion superoxide production which is dismuted in H$_2$O$_2$. Other studies have shown that increased FFA in type 2 diabetes induce the long-chain acyl-CoA accumulation and diacylglycerols which are potent activators of protein kinase C isoforms and the kappa B nuclear factor [7, 8]. All mechanisms of kinase activation may explain the ROS formation mediated by FFA [9]. Other studies have shown that prolonged exposure of islets to fatty acids induces the production of peroxynitrite (ONOO$^-$), which bind to cytochrome C oxidase and inhibit the respiratory chain. Permeability transition pore is open, leading to a proton leakage and an ATP hydrolysis. Mitochondria swell and release the cytochrome C, which activates cytoplasmic proteases called caspas. These are responsible for proteolysis stimulation and cell death [10]. However, a recent study suggests that beta mitochondrial oxidation cannot fight against the FFA increasing, in particular palmitic acid, which is associated with obesity and type 2 diabetes. This overload is conveyed to the peroxisomal beta-oxidation leading to H$_2$O$_2$ production [11]. In this study we found a positive correlation between homocysteine and H$_2$O$_2$ (Fig. 3, r = 0.5, p<10$^{-5}$). Peyrin-Biroulet et al. showed in vitro that homocysteine has a pro-oxidant action; the thiol group is oxidized to form ROS [12].

### III. Results

The H$_2$O$_2$ concentration determined was four times higher in type 2 diabetes compared with controls. A positive correlation was found between HbA1c and H$_2$O$_2$ concentration (r = 0.85, p<10$^{-3}$, Figure 1). Another positive correlation was observed between FFA concentration and H$_2$O$_2$ concentration.

### Table 1

Characteristics of the patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HDP (n=26)</th>
<th>NDP (n=22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>54.3±11.7</td>
<td>50.4±10.4</td>
<td>0.23</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>12/14</td>
<td>15/7</td>
<td>0.12</td>
</tr>
<tr>
<td>Duration of diabetes (months)</td>
<td>72 [48, 120]</td>
<td>68 [36, 120]</td>
<td>0.7</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.6 ± 1.4</td>
<td>8.2±1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Duration of hypertension (months)</td>
<td>84 [60, 120]</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>25.3±4.1</td>
<td>24.9±3.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Basal SBP (mmHg)</td>
<td>150.3±17.9</td>
<td>113.1±12.6</td>
<td>0.001</td>
</tr>
<tr>
<td>Basal HR (beats/min)</td>
<td>77.5±15.2</td>
<td>78.2±14.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Values expressed as mean± SE. p significant if < 0.05. Student’s t-test.

HbA1c: Glycated hemoglobin A$_1$C
BMI: Body mass index
SBP: Systolic blood pressure
HR: Heart rate
* Median and quartiles
Figure 1: Positive correlation between glycated hemoglobin (HbA1c) and hydrogen peroxide concentration (H$_2$O$_2$); r=0.85 and p<10$^{-3}$. Error bars show 95% CI of mean.

Figure 2: Positive correlation between free fatty acids concentration (FFA) and hydrogen peroxide concentration (H$_2$O$_2$); r=0.9 and p<10$^{-3}$. Error bars show 95% CI of mean.

At high concentrations, homocysteine undergoes auto-oxidation producing homocysteine and superoxide anion (O$_2^-$) [13]. This activates the superoxide dismutase and reduced to H$_2$O$_2$.

Moreover, homocysteine binds to proteins, producing ROS. These changes can affect LDL protein contributing to their retention in the intima. Recently, it has been shown that homocysteine increases mitochondrial H$_2$O$_2$ production in the kidney [14].

We found a negative correlation between QUICKI and H$_2$O$_2$ (Figure 4, r = 0.5, p<10$^{-3}$). This reflects that chronic hyperglycemia increases the resistance of peripheral tissues to insulin via H$_2$O$_2$ in type 2 diabetes. A recent study has shown that insulin resistance is associated with an increased capacity for mitochondrial H$_2$O$_2$ release in Zucker diabetic fatty rats [15]. Moreover, another report has discerned that insulin increased ROS production by pancreatic beta cells and also increased cell death when treated by H$_2$O$_2$. These effects were accentuated by the inhibition of receptor signaling to insulin,
which indicated an independent effect of the waterfall insulin resistance. This study concludes that high levels of insulin may exacerbate cell death induced by H\textsubscript{2}O\textsubscript{2} and other apoptosis inducers [16]. However, several experiments on cell lines in vitro have shown that oxidative stress inhibits signal transduction of insulin. H\textsubscript{2}O\textsubscript{2} micromolar concentrations inhibit the auto-phosphorylation of insulin receptor, the insulin receptor substrate-1 (IRS-1) and the downstream events of IRS-1, including the activation of phosphatidylinositol 3-kinase and glucose transport, and the activation of mitogen-activated protein kinases (MAPK) [17].

Oxidative stress inhibits the glucose transporter GLUT4 translocation [18] and the protein kinase C activation [19] stimulated by insulin in fat cells. Exposure of cell cultures for 2 h at 60-90 uM of H\textsubscript{2}O\textsubscript{2} has been shown to cause a significant loss of insulin stimulation in both proximal (IR tyrosine phosphorylation) and distal (Akt and GSK-3β phosphorylation of serine) elements of insulin signaling and glucose transport activity [20]. Exposure of isolated soleus muscle for 4 h at the same concentration of H\textsubscript{2}O\textsubscript{2} was associated with a selective loss of IRS-1 and IRS-2, exacerbating the loss of insulin in response to oxidative stress [21].

We found 2 additional correlations: a correlation between H\textsubscript{2}O\textsubscript{2} concentration and arterial hypertension (r=−4, p<10\textsuperscript{-3}) and a correlation between homocysteine and arterial hypertension (r=−7 p<10\textsuperscript{-3}). These results allow assuming that homocysteine affect endothelial cell function via H\textsubscript{2}O\textsubscript{2}. Overproduction of H\textsubscript{2}O\textsubscript{2} may change the signal transduction machinery and the regulation of gene expression, causing an imbalance between proliferation, hypertrophy and apoptosis of smooth muscle cells and endothelial dysfunction involved in the pathogenesis of atherosclerosis and restenosis. Our results are partially consistent with those of Framingham Heart Study 5 [22]. Indeed, they are in favor of a link between homocysteine concentrations and increased cardiovascular risk. This association was more pronounced among women. On the other side, no relationship was found between homocysteine and arterial hypertension. Many experimental and clinical studies have also shown a major role of the deficiency in omega-3 and excess saturated fatty acids, oxidative stress including cell membranes and disorders of methylation reactions resulting in hyperhomocysteinemia [23]. One study showed that mice with type 2 diabetes have a significantly increased production of H\textsubscript{2}O\textsubscript{2} in the arteriolar wall [24]. Besides, other than its inhibitory action on endothelial NO production, H\textsubscript{2}O\textsubscript{2} can actively participate in the mechanisms of endothelium-dependent vasodilatation in type-2 diabetes. The mechanism of H\textsubscript{2}O\textsubscript{2}-mediated dilatation is not fully understood, but studies have shown that following the stimulation of potassium channels by calcium, H\textsubscript{2}O\textsubscript{2} hyperpolarizes the vascular smooth muscle cells and acts as a potential endothelium-derived hyperpolarizing factor [25, 26, 27]. Other studies have shown that the vasodilatation, induced by H\textsubscript{2}O\textsubscript{2}, is mediated by endothelial NO release [28]. In vitro, studies have found that during the exposure of human endothelial cells from umbilical veins at low glucose concentrations (up to 0.4 g / l); NO levels lowered quickly in response to decreased synthesis, by eNOS, and accelerated degradation. This was accompanied by activation of O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} production, which are coupled by the mitochondrial membrane hyperpolarization due to the lack of NO [29].

V. CONCLUSIONS

The overproduction of H\textsubscript{2}O\textsubscript{2} generated by hyperglycemia, increased dose of FFA and hyperhomocysteinemia, and amplified the insulin resistance.

REFERENCES


