

## Oxidative Stress, Diabetes, and Its Complications

a report by

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Our evolution as complex aerobic organisms with high energy demands is tightly linked to the acquisition of mitochondria in our cells. This passage gave us the ability to use oxygen, harnessing its power in the respiratory chain, but also the fundamental function to compartmentalize this process, protecting the cytosol from its potentially harmful side effects.<sup>1</sup> In fact, even being scarcely reactive this molecule has the tendency to 'radicalize,' forming incompletely reduced molecules characterized by an uncoupled electron. This highly reactive and short-living class of molecules is known as reactive oxygen species (ROS). ROS production is a side effect of the normal metabolism of the cell, which developed a series of enzymes able to disarm them. Superoxide dismutase, catalase, and glutathione peroxidase are powerful weapons that act together with antioxidant molecules introduced with diet to protect the organism. In healthy subjects there is a balance between ROS formation and elimination. Every time this balance is lost due to an augmented production of reactive species or due to a reduction in antioxidant production or activity there is a condition of oxidative stress. Losing control of ROS is very harmful and almost all the constituents of the cell can be targets of these molecules. DNA, proteins, and lipids can be involved in chain reactions that entail their modification and, in the worst case, the loss of their functionality. Genetic degeneration and physiological dysfunction can lead to cell death and aging of the organism.

On this basis it is not surprising that oxidative stress has been implicated in

a growing list of human diseases with a leading place occupied by diabetes for two reasons, the first being the epidemic proportions that this disease is assuming. Numbers are increasing: in 2003, people with diabetes numbered 197 million worldwide, rising to 333 million by 2025, with six million new cases every year.<sup>2</sup> This means that every 10 seconds one person dies of diabetes-related diseases and in the same 10 seconds two people develop diabetes. The second reason is the unifying hypothesis that recent studies propose to explain the rise of diabetic complications, and that assign a leading role to oxidative stress.<sup>3</sup>

Fighting diabetic complications will be the goal over the next few years. In fact, at the present time, insulin and other drugs can help to control many aspects of diabetes. However, the good news represented by the ability to control hyperglycemic episodes was jeopardized by the increased risk of developing many serious complications involving the vascular system, the kidneys, and the nervous system. These complications are frequent and costly in terms of longevity and quality of life. Diabetes is the prevalent cause of blindness in adults of working age, end-stage renal failure requiring dialysis or transplantation, and non-traumatic amputation, and it is also an important factor in the etiology of heart attack and stroke.<sup>4</sup>

The unifying hypothesis could be an important step in the way to control and fight these complications. However, in order to completely appreciate the elegance of this hypothesis that, as one of its main contributors Michael Brownlee said, "put together the previous theories like the pieces of a puzzle",<sup>3</sup> we have to consider our knowledge of the problem.

Four major molecular damage pathways were recognized to be distinctive of the diabetic condition: the polyol pathway, the increased formation of advanced glycation end-products (AGEs), a hyperglycemia-induced activation of protein kinase C (PKC), and an increased hexosamine pathway flux.

The polyol pathway synthesizes fructose from glucose in a series of passages. In the first of these, the enzyme aldose reductase reduces glucose to the sugar alcohol sorbitol, using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. The low affinity of aldose reductase for its substrate means that only a low percentage of glucose takes this pathway in normal euglycemic conditions.<sup>5</sup> However, in the diabetic patient high glucose levels in the blood determine an augmented production of sorbitol with a concomitant decrease in the NADPH pool.<sup>6</sup> There is an overstocking of sorbitol inside the cell with an augmented osmotic pressure and a reduced availability of the antioxidant glutathione (GSH), which needs NADPH for its regeneration. The second pathway



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involved in diabetic complications entails the intracellular formation of AGEs. The augmented presence of glucose inside the cell originates reactive dicarbonyl molecules such as glyoxal, methylglyoxal, and 3-deoxyglucosone, which react with the aminic groups of proteins to form AGEs. The modification process does not require the presence of an enzyme and the two-step reaction is not reversible. Proteins with a very slow turnover rate, such as collagen and hemoglobin, exist in the body partly modified by glucose. These kind of modifications usually imply a loss of functionality and the denaturation of the target protein; moreover, the binding of the modified protein to AGE receptors on endothelial cells, mesangial cells, and macrophages induces the production of reactive oxygen species. AGEs are proved to be responsible for several aspects of diabetic complications involving vessels and kidneys.<sup>7,8</sup>

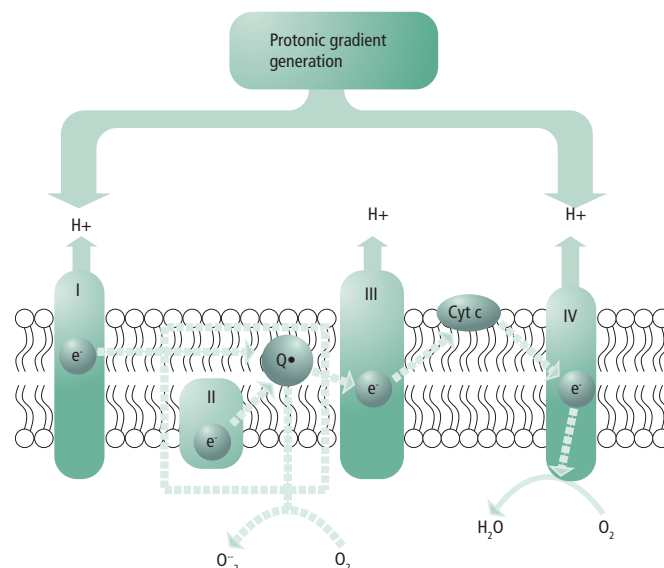
PKC is a family of at least 11 serin/treonin protein kinase isoenzymes involved in several cellular responses, such as growth, differentiation, genic expression, angiogenesis, and sorting of proteins inside the cell's district. Based on their activating substances, the isoenzymes are classified in different families. The conventional PKCs require calcium and diacylglycerol (DAG) for activation, while the new PKCs require DAG but are calcium-independent.<sup>9</sup> In diabetic patients, the augmented availability of glucose causes an augmented availability of DAG and a consequent activation of PKCs. Effects of this extra-activation are different and detrimental for vascular functionality with increased vascular permeability,<sup>10</sup> deregulated nitric oxide generation via NADPH oxidase activation,<sup>11</sup> stabilized vascular endothelial growth factor (VEGF) messenger ribonucleic acid (mRNA)

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expression through post-transcriptional mechanisms, increased leukocyte-endothelium interaction, and activating nuclear factor kappa Beta (NF-kB).<sup>12</sup>

Last but not least, in diabetes there is an augmented flux through the hexosamine pathway (HP). In the healthy metabolism a relatively low amount of fructose-6P is diverted from glycolysis and directed to a cascade of reactions whose end-product is a series of amino-sugars, the building blocks of the glycosyl-side chains of proteins and lipids.<sup>13</sup> Unlike AGE formation, the modification of proteins and lipids requires specific enzymes. Once again, the augmented availability of glucose in the diabetic patient causes an accumulation of end-products of this pathway. This was associated with the development of the complications of diabetes, basically due to the modification on serine/threonine residues of selected proteins.<sup>14-16</sup> HP was involved in transforming growth factor beta-1 (TGF- $\beta$ 1) and fibronectin expression,<sup>17,18</sup> and stimulates plasminogen activator

**Figure 1: Superoxide Anion Production at the Mitochondrial Electron Transport Chain Level**



inhibitor 1 (PAI-1) via modification of the transcription factor stimulatory protein 1 (Sp1).<sup>19</sup> Augmented flux through the HP was also linked to insulin resistance and several authors proposed that flux through the hexosamine pathway may represent a cellular 'sensor' to monitor the flux of a nutrient, glucose, that can become toxic when in excess.<sup>20</sup> This position ascribes to insulin resistance a sort of protective action to minimize glucose entrance.

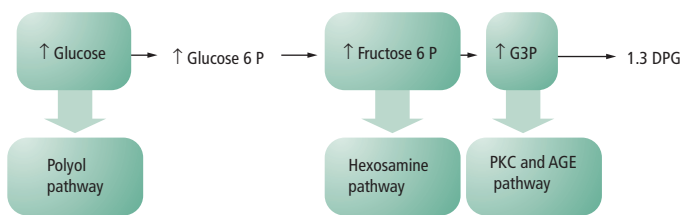
So, there are four apparently distinct pathways whose end-points are diabetic complications, apparently because the unifying theory proposes a unique activating event for all of them: an upstream event able to start all the damaging pathways. This event is the production of ROS at the mitochondrial electron transport chain in high-glucose condition. It has been proved that there exists a threshold protonic potential, above which electron transfer between the complexes of the transport chain is inhibited and a very strong increase in ROS production takes place.<sup>21</sup> The electrons, being forbidden the natural transfer, are given up to the molecular oxygen, originating superoxide anion (see *Figure 1*).

In an elegant series of experiments, complex two of the mitochondrial electron transport chain was proved to be the site of superoxide generation, and it was established that the inhibition of the production of superoxide anion from this site is enough to block the activation of all four damaging pathways discussed above.<sup>22</sup> In fact, the same substances effective in preventing mitochondrial ROS formation also inhibited PKC activation, intracellular AGE formation, and polyol pathway flux, preventing sorbitol accumulation. Superoxide production and oxidative stress generation is only the first step; in the following years, the mechanism by which the superoxide produced at the electron transport chain was able to activate the damage pathways was pinpointed.

Recently, Du and colleagues demonstrated that in aortic endothelial cells cultured in 5mM glucose, inhibition of the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) enzyme using antisense oligonucleotides activated

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**Figure 2: Effects of GAPDH Inhibition on the Four Major Hyperglycemic Damage Pathways**



the same damaging pathways that were activated in cells cultured in 30mM glucose. Afterwards, it was corroborated that the cascade of events leading to GAPDH inactivation in high-glucose-treated cells started with ROS production at the mitochondrial level, and it was suggested that the oxidative stress so generated determined DNA strand breaks and the activation of the poly (ADP-ribose) polymerase enzyme that modified GAPDH inhibiting it.<sup>23</sup>

It is not difficult to imagine the effect of inhibiting this key enzyme of glycolysis. In a cell environment stressed by the presence of high glucose concentration, the main pathway of utilization of this molecule becomes impeded. The substrate is then addressed to all the other pathways of utilization, which are the damaged pathways considered above. The low affinity for its substrate of aldose reductase was overtaken by the high glucose presence; in the same way, both the enzymatic and the non-enzymatic glycosylation process were boosted and the augmented availability of DAG causes the activation of the DAG-sensitive isoforms of PKC (see *Figure 2*).

As we have previously considered, activation of these pathways is reflected in an augmented oxidative stress inside the cell. The presence of sorbitol, a molecule that can hardly pass through membranes, influences the osmotic

pressure in the cell, but mainly shoots down cell antioxidant defense subsiding its GSH pool. Once produced, AGEs can react with specific receptors for advanced glycation end-products (RAGEs) on endothelial cells, mesangial cells, and macrophages in a process that has been proved to mediate signal transduction through generation of reactive oxygen species, and that lead to activation of both the transcription factor NF-κB and farnesyl-protein transferase (p21<sup>ras</sup>).<sup>24,25</sup>

Not all the passages have been clearly elucidated, but evidence is amassing and the role of oxidative stress is changing from that of a walker-on to a main character. Understanding the entire cascade of events would give us the capacity to fight diabetic complications at their early beginning, developing

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new molecules that are more specific and effective. At the moment this is still not possible, even if some pleasant secret is hidden by some drugs already in use. It is now well recognized that thiazolidinedones,<sup>26</sup> statins,<sup>27,28</sup> ACE inhibitors, and angiotensin-1 (AT-1) inhibitors<sup>29</sup> have shown beneficial ‘unpredicted’ effects exceeding expectations. Different authors are oriented to ascribe these effects to the antioxidant activity that is now recognized in these molecules.

So, a lot of work has still to be done, but the road seems fair and the first steps of this long trip were very promising. ■

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