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Na^+/K^+ ATPase Impairment and Experimental Glycation: the Role of Glucose Autoxidation

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Non enzymatic glycation could be involved in the early impairment of Na^+/K^+ ATPase that occurs in sciatic nerve of diabetic rats. In fact, decrease of Na^+/K^+ ATPase activity is one of the first alterations showed in experimental diabetic neuropathy. In this respect, it is known that in the presence of transition metals under physiological conditions, glucose can autoxidize yielding hydrogen peroxide (H_2O_2) and free radical intermediates, which, in turn, inhibit the cation pump. Our experiments were designed to determine if glucose autoxidation has any relevance in the early steps of Na^+/K^+ ATPase experimental glycation. Compared experiments with and without the sodium borohydride ($NaBH_4$) reduction step demonstrated that incubation of brain Na^+/K^+ ATPase with glucose 6-phosphate (G 6-P) and trace metals induced a significant decrease in enzyme activity dramatically enhanced by addition of copper (Cu^{2+}). A concomitant production of H_2O_2 was noticed. The presence of diethylenetriaminepentaacetic acid (DTPA), a strong metal chelator, completely prevented Na^+/K^+ ATPase impairment and hydrogen-peroxide formation. No gross structural and conformational alterations of the enzyme can be demonstrated by intrinsic and extrinsic fluorescence measurements. Our results suggest that during the exposure of brain Na^+/K^+ATPase to glucose 6-phosphate in vitro (experimental glycation), the decrease in activity can be correlated, at least in the early phases, to metal-catalyzed production of oxidative species, such as H_2O_2, through the glucose autoxidation process, and not to glucose attachment to the enzyme. Since plasma hydroperoxides and copper appear to be elevated in diabetic patients with complications, our data suggest a critical role for oxidative reactions in the pathophysiology of the chronic complications of diabetes like neuropathy.

Key words: Na^+/K^+ ATPase, glucose autoxidation, experimental glycation, hydrogen peroxide, metals

INTRODUCTION

The exposure of proteins to glucose *in vitro* (experimental protein glycation) has been widely used as a model for tissue damage associated with diabetes mellitus.[1-6] The covalent attachment of

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glucose to amino groups alone is no longer sufficient to account for structural changes observed during such *in vitro* studies.[5–7] In fact, it has been suggested that transition metal-catalyzed glucose enediol oxidation (glucose autoxidation) could be a factor responsible for protein modifications associated with experimental glycation.[7–11]

In animal model, a reduction of Na^+/K^+ ATPase activity is postulated to play a pivotal role in the pathogenesis of experimental diabetic neuropathy.[12,13] The impairment of the cation pump is supposedly related to a derangement of phosphoinositide turnover (which in turn may result from alteration in myoinositol uptake and metabolism) and/or to an activation of the polyol pathway.[12] Neither of these proposed schemes addresses the fact that Na^+/K^+ ATPase may be inhibited by the attachment of glucose to critical amino groups of the enzyme, which play an important role for both Na^+ and K^+ interactions, or by the action of hydrogen peroxide, generated by glucose autoxidation, which has already been demonstrated to decrease cation pump activity in the ischemia-reperfusion injury of the kidney.[14,15]

The present study was therefore undertaken to assess the contribution of glucose autoxidation to experimental glycation of brain Na^+/K^+ ATPase, and to have a better insight on the role of oxidative reactions in the pathophysiology of the chronic complications of diabetes. Even if the β subunit of Na^+/K^+ ATPase can be glycated, this would not affect our experimental model since all the known catalytic functions of Na^+/K^+ ATPase are associated with the α subunit and have been tentatively related to distinct segments of the polypeptide.[2,3,16]

MATERIALS AND METHODS

Chemicals

Na^+/K^+ ATPase from porcine cerebral cortex, xylenol orange, H_2O_2, eosin-Y, diethylenetriaminepentaacetic acid (DTPA), $CuSO_4$, $CuCl_2$ and $MgCl_2$ were obtained from Sigma (St. Louis, Mo., USA). Na_2ATP and glucose 6-phosphate (sodium salt) (G6-P) were purchased from Boehringer Mannheim (Mannheim, Germany). Sodium borohydride (99%) ($NaBH_4$) was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were of the highest quality available.

Brain Na^+/K^+ ATPase, free of ouabain-insensitive ATPase activity, was dissolved in 30 mM imidazole buffer, pH 7.4, containing 150 mM KCl. Sucrose and EDTA, contained in the commercial preparation, were removed by ultrafiltration through a Diaflo YM 10 membrane, Amicon Corporation (Danvers, MA, USA).

Incubation procedure

Na^+/K^+ ATPase (0.7 mg/ml) was incubated with 20 mM glucose 6-phosphate at 37°C for 2 hours in the dark, according to Garner *et al.*[2,16] The use of glucose 6-phosphate is strongly indicated because the phosphate residue accelerates the rate of glycation of the enzyme, which is not very stable at 37°C for a long time,[3] without bringing any difference in the type of product observed. In selected experiments, 1 mM DTPA and/or 20 μM Cu^{2+} (chloride and/or sulphate salts) were added. After two hours, 100 mM $NaBH_4$ in 0.5 M histidine was added to the reaction mixture. Sodium borohydride provides a reducing environment which greatly increases the rate of glucose attachment to the amino groups and hence the Na^+/K^+ ATPase glycation. Sodium borohydride reduction is known to have no adverse effect on Na^+/K^+ ATPase.[16] In selected experiments the borohydride reduction step was omitted and simple 0.5M histidine was added to the reaction mixture. The borohydride reduction proceeded for 60 min. The Na^+/K^+ ATPase was isolated from this mixture by centrifugation at 100 000 × g for 1 h. The pelleted enzyme was rinsed twice by resuspension in 1 mM imidazole, pH 7.4, and recentrifuged for 1 h at 100 000 × g. The supernatant was discarded and the pelleted enzyme was resuspended in 0.3 ml of 30 mM imidazole, 150 mM KCl, pH 7.4. Control samples were prepared with an identical

procedure except for elimination of glucose 6-phosphate.

Evaluation of enzyme glycation

The enzyme glycation was evaluated with the affinity-chromatografic method,[17,18] using the GLYCO GEL Test kit, Pierce (Rockford, IL, USA). It consists of prepacked 1-ml columns of cross-linked, beaded agarose which incorporates an immobilized ligand (*m*-aminophenyl boronic acid) capable of specifically interacting with non-enzymatically glycosylated proteins. The amount of sugar bound to the enzyme, following the incubation with G6-P, $NaBH_4$, Cu^{2+} and/or DTPA, was determined, at room temperature, adding 200 µl aliquots of Na^+/K^+ ATPase (in 30 mM imidazole, 150 mM KCl, pH 7.4) to the top disc of the prepacked columns. Then, 20 ml of wash buffer (250 mM ammonium acetate and 50 mM magnesium chloride, pH 8.0) were added, and the total collected eluate (20.2 ml) comprised the not-bound, non-glycosylated enzyme. Next, 3 ml of elution buffer (150 mM sodium citrate) were added and the entire fraction (containing the bound, glycosylated enzyme) was collected. The percentage of glycosylation of different aliquots of Na^+/K^+ ATPase was calculated measuring the absorbance at 280 nm, according to the formula

$$\%\ \text{Glycated enzyme} = \frac{3.0 \times A_{280}\ \text{bound/glycated}}{20.2 \times A_{280}\ \text{non-bound/non-glycated} + 3.0 \times A_{280}\ \text{bound/glycated}} \times 100$$

and determining protein concentration of different aliquots by the method of Bradford.[19]

Measurement of Na^+/K^+ ATPase Activity

Measurement of enzyme activity was performed following the hydrolysis of ATP at 37°C in 30 mM histidine buffer, pH 7.4, containing 130 mM NaCl, 20 mM KCl, and 4 mM $MgCl_2$, according to the coupled assay of Norby[20]. The ATPase reaction was started by the addition of 3 mM Na_2ATP. The results have been expressed as micromoles of P_i per milligrams of protein per hour.

Measurement of hydrogen peroxide production

The production of H_2O_2 following incubation of Na^+/K^+ ATPase with glucose-6 phosphate or Cu^{2+} was determined according to the FOX assay of Wolff *et al.*[8,9]. This technique relies upon the rapid hydrogen peroxide-mediated oxidation of Fe^{2+} under acidic conditions. Fe^{3+} forms a chromofore with xylenol orange which absorbs strongly at 560 nm. After incubation of the enzyme in different conditions and the reduction step, 100 µl samples were added to 900 µl of reagent (100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 25 mM H_2SO_4 and 100 mM sorbitol). Absorbance was read at 560 nm after 45 min incubation at room temperature, following a 2 min centrifugation at 12 000 × g to remove any flocculated protein. Catalase (100 U/ml) was added to compared samples, prior to the FOX reagent, to be sure that H_2O_2 was being measured. The signal was read against a H_2O_2 standard curve. DTPA present in the incubation mixture, which could interfere with the FOX assay, was completely removed washing twice the pelleted enzyme (see *Incubation Procedure*).

Conformational studies

Fluorescence studies were performed on a LS5 Luminometer (Perkin-Elmer Beaconsfield, Bucks., U.K.). 30 nM eosin Y was dissolved in 20 mM histidine-HCl, pH 7.4: the excitation maximum was 518 nm and the emission maximum 538 nm. Then 0.1 mg/ml Na^+/K^+ ATPase (incubated in different conditions) were added, to verify the increase in fluorescence and the appearance of a shoulder on the excitation curve around 490 nm, which is the effect of the addition of the enzyme in the E_1 conformation.[21–23]

Glycofluorophores, the novel fluorophores formed as result of the exposure of protein to glucose, were measured using excitation at 350 nm and emission at 415 nm.[1,5] Formation of

advanced glycosylation endproducts (AGEs) was followed with 370 nm excitation and 440 nm emission.[1,5,7] Intrinsic fluorescence was monitored with excitation at 280 nm. In all the experiments the enzyme (0.1 mg/ml) was in 30 mM imidazole, 150 mM KCl, pH 7.4.

To verify if the magnesium binding site of Na^+/K^+ ATPase was involved in the glycation/glucose autoxidation process, the enzyme (0.7 mg/ml) was preincubated with 3 mM $MgCl_2$ for 20 min at 25°C, before the usual incubation conditions.

RESULTS

The results of incubation of brain Na^+/K^+ ATPase with glucose 6-phosphate, Cu^{2+} and/or DTPA, followed by $NaBH_4$ reduction and hence enzyme glycation, are shown in Table 1. The incubation of Na^+/K^+ ATPase with 20 mM glucose 6-phosphate provoked a 28% decrease in the enzyme activity ($p < 0.001$) with respect to the enzyme incubation in simple buffer. Addition of 20 μM Cu^{2+} to the incubation mixture, led to a dramatic fall in the cation pump activity of 85% ($p < 0.001$). Addition of DTPA to the incubation mixture not only prevented the cation pump impairment by glucose 6-phosphate and trace metals, but also protected the enzyme from the coupled inactivating action of glucose 6-phosphate and added copper. A glucose 6-phosphate concentration of 5 mM had no effect as had the simple incubation with DTPA (data not shown).

The simple incubation of Na^+/K^+ ATPase with glucose 6-phosphate, without $NaBH_4$ reduction (see again Table 1), determined a decrease of 24% ($p < 0.001$) in the enzyme activity, while the addition of 20 μM Cu^{2+} to the incubation mixture led to a dramatic fall in the cation pump activity (82%), very similar to that observed in the presence of $NaBH_4$. Also in this case, addition of DTPA to the incubation mixture not only prevented the cation pump impairment by glucose 6-phosphate and trace metals, but also shielded the enzyme by the coupled inactivating action of glucose 6-phosphate and copper. It is worthwhile to mention that incubation of the enzyme with only copper (without glucose 6-phosphate) induced a decrease of 8% ($p < 0.01$) in Na^+/K^+ ATPase activity.

As shown in Table 2, monitoring glycation of Na^+/K^+ ATPase with affinity chromatography indicates a significant incorporation of glucose 6-phosphate into the enzyme molecule, and hence glycation, only when the Schiff base has been stabilized by borohydride reduction. Furthermore, no statistically significant differences in the enzyme glycation were observed when Cu^{2+} and/or DTPA were added to the incubation mixture.

Hydrogen peroxide production, during incubation of brain Na^+/K^+ ATPase with 20 mM glucose 6-phosphate, Cu^{2+} and/or DTPA, followed

TABLE 1 Effect on Na^+/K^+ ATPase activity of different incubation conditions, followed or not by $NaBH_4$ reduction, as described in METHODS

	Na^+/K^+ ATPase activity (μmol P_i/mg protein/hour)	
incubation with:	+$NaBH_4$	–$NaBH_4$
buffer	41.75 ± 0.78	41.78 ± 1.38
G 6-P	30.02 ± 0.82*	31.78 ± 0.63*
G 6-P + Cu^{2+}	6.35 ± 0.51*	7.81 ± 0.63*
G 6-P + DTPA	41.51 ± 1.12	40.80 ± 1.19
G 6-P + Cu^{2+} + DTPA	41.89 ± 1.43	41.89 ± 1.63
+ Cu^{2+}		38.31 ± 0.45**

Data are means ± SD. Comparisons are made between enzyme incubated with buffer and enzyme incubated in different conditions. * $p < 0.001$, ** $p < 0.01$.

TABLE 2 Evaluation of Na^+/K^+ ATPase glycation following different incubation conditions, as described in METHODS

	% glycated Na^+/K^+ ATPase	
incubation with:	+$NaBH_4$	–$NaBH_4$
G 6-P	99.87	0.02
G 6-P + Cu^{2+}	99.75*	0.03*
G 6-P + DTPA	99.49*	0.01*
G 6-P + Cu^{2+} + DTPA	99.83*	0.02*

Comparisons are made between enzyme incubated with G 6-P and enzyme incubated with Cu^{2+} and/or DTPA. *p = NS.

by the borohydride reduction step, is shown in Table 3. Glucose 6-phosphate alone slightly stimulated peroxide formation: 1.71 ± 0.13 μM ($p < 0.001$ vs the enzyme incubated with buffer). When Cu^{2+} and glucose 6-phosphate were both included in the incubation mixture, the effect was much greater: 6.06 ± 0.15 μM ($p < 0.001$ vs the enzyme incubated with buffer). On the contrary, addition of DTPA completely prevented hydrogen peroxide production in both cases.

The simple incubation of Na^+/K^+ ATPase with glucose 6-phosphate or glucose 6-phosphate and Cu^{2+} (see again Table 3) led to the production of hydrogen peroxide in quantities very similar to those obtained when the borohydride reduction step was performed. Even in these cases addition of DTPA completely prevented hydrogen peroxide production. Incubation of the enzyme with only copper (without glucose 6-phosphate) induced a small but significant hydrogen peroxide formation ($p < 0.01$).

With eosin Y as a conformational probe, the incubation with glucose 6-phosphate or glucose 6-phosphate plus copper, followed by the borohydride reduction step, produced no conformational modification of Na^+/K^+ ATPase in terms of both extrinsic fluorescence spectra, which were completely overlapping, and appearance of a shoulder on the excitation curve around 490 nm (data not shown). The same results were obtained with the simple incubation with glucose 6-phosphate or glucose 6-phosphate plus copper.

Furthermore, as shown in Table 4, following incubation in different conditions, Na^+/K^+ ATPase intrinsic fluorescence emission maxima did not show any modification and there was not any increase in non-tryptophan fluorescence, so excluding any formation of glycofluorophores or advanced glycosylation endproducts, AGEs.

As shown in Table 5, the preincubation of the Na^+/K^+ ATPase with $MgCl_2$ did not prevent the decrease of enzymatic activity following the different incubation conditions.

DISCUSSION

Although the role of oxidation reactions and metal ion catalysts in the Maillard reaction is widely

TABLE 3 Production of hydrogen peroxide by glucose autoxidation during incubation of Na^+/K^+ ATPase with 20 mM glucose 6-phosphate in different conditions, as described in METHODS

	H_2O_2 (μM)			
incubation with:	+$NaBH_4$		–$NaBH_4$	
	–Catalase	+Catalase	–Catalase	+Catalase
buffer			0.003 ± 0.001	0.002 ± 0.001
G 6-P	1.41 ± 0.11*	0.09 ± 0.01*	1.71 ± 0.15*	0.11 ± 0.02*
G 6-P + Cu^{2+}	5.79 ± 0.12*	0.40 ± 0.08*	6.06 ± 0.15*	0.42 ± 0.03*
G 6-P + DTPA	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001
G 6-P + Cu^{2+} + DTPA	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001
+ Cu^{2+}			0.114 ± 0.007**	0.008 ± 0.002

Data are means ± SD. Comparisons are made between enzyme incubated with buffer and enzyme incubated in different conditions. *$p < 0.001$, **$p < 0.01$.

TABLE 4 Effect of different incubation conditions on Na^+/K^+ ATPase intrinsic fluorescence, glycofluorophores formation and advanced glycosylation endproducts (AGEs) formation. Fluorescence was measured at the end of different incubation conditions, as described in METHODS

	Intrinsic Fluorescence %		Glycofluorophores Formation %		AGEs Formation %	
	+$NaBH_4$	−$NaBH_4$	+$NaBH_4$	−$NaBH_4$	+$NaBH_4$	−$NaBH_4$
incubation with:						
buffer	100	100	0	0	0	0
G 6-P	98.5	99.1	2.1	2.3	1.3	2.1
G 6-P + Cu^{2+}	97.9	98.1	2.2	1.3	1.8	1.5
G 6-P + DTPA	99.2	97.8	2.3	1.4	2.1	1.9
G 6-P + Cu^{2+} + DTPA	98.7	99.1	1.4	2.1	1.6	2.4
+ Cu^{2+}	98.1	98.5	1.1	1.2	1.3	1.6

Data are representative of results from three experiments. Comparisons are made between enzyme incubated with buffer and enzyme incubated in different conditions. In all cases, p = NS.

recognized, the sequence of the reactions, i.e. glycation versus oxidation, is still uncertain. Our results suggest that the decrease in Na^+/K^+ ATPase activity observed after the exposure of brain Na^+/K^+ ATPase to glucose 6-phosphate *in vitro* can be correlated, in the early phases, to metal-catalyzed production of oxidative species, such as H_2O_2, through the glucose autoxidation process, rather than to glycation.

In fact, as shown in Table 1, the glycated enzyme (following reduction with $NaBH_4$) shows a reduced activity very similar to that of the non-glycated enzyme. Our observation that reduction with $NaBH_4$, and hence glycation, induces a slightly higher decrease in the enzyme activity (see again Table 1) only apparently conflicts with earlier reports on the ability of reducing agents in preventing glycoxidative processes.[24] In fact, in our experiments $NaBH_4$ was added after two hours incubation of Na^+/K^+ ATPase with glucose and copper and hence it could not prevent enediol and protein-enediol formation and the production of dicarbonyl products.[10,24] Furthermore, $NaBH_4$ is a less selective reducing agent than $NaCNBH_3$ and further oxidative steps of the Maillard reaction cannot be excluded,[1,5,7,25] since the Amadori product autoxidation is a process occurring at physiological pH.[7,11]

The Na^+/K^+ ATPase activity decrease observed after the incubation with only 20 mM glucose 6-phosphate, followed or not by $NaBH_4$ reduction, is to be related to the presence of trace metals in the incubation buffer, which catalyze the glucose autoxidation process, and not to glycation. In fact, addition of DTPA, a strong metal chelator, to the incubation mixture completely prevents the cation pump impairment (Table 1). The dramatic fall in cation pump activity, observed in parallel

TABLE 5 Effect of magnesium preincubation on Na^+/K^+ ATPase activity in different incubation conditions, as described in METHODS.

	Na^+/K^+ ATPase activity (μmol P_i/mg protein/hour)	
incubation with:	+$NaBH_4$	−$NaBH_4$
buffer	42.88 ± 0.92	42.34 ± 1.22
G 6-P	31.12 ± 0.78*	33.12 ± 0.75*
G 6-P + Cu^{2+}	7.27 ± 0.47*	9.12 ± 0.56*
+ Cu^{2+}		39.27 ± 0.39**

Data are means ± SD. Comparisons are made between enzyme incubated with buffer and enzyme incubated in different conditions. *p<0.001, ** p<0.01.

experiments with the addition of copper to the incubation mixture, is due to the combined action of glucose 6-phosphate and the metal which enhance glucose autoxidation and production of oxidizing intermediates. Also in this case addition of DTPA to the incubation mixture completely prevented cation pump impairment. The fact that the percentage of glucose attachment to the enzyme (i.e. glycation) did not vary in the different incubation conditions (Table 2) excludes a direct effect of DTPA on the glycation process and a modification of glycation sites by the oxidative species. On the contrary, DTPA could prevent Na^+/K^+ ATPase impairment by inhibiting the metal-catalyzed process of glucose autoxidation and the production of hydroxyl radical from hydrogen peroxide.[7,10,25] Copper could induce enzyme inactivation with a double mechanism: 1) catalyzing glucose autoxidation, with H_2O_2 production,[11,24] and 2) stimulating hydroxyl radical production from accumulated hydrogen peroxide through a Fenton's reaction.[7,10,11,24]

The role of oxidation in enzyme inactivation is further supported by the production of hydrogen peroxide seen in parallel experiments, which was only slightly enhanced by the enzyme glycation (Table 3). Glucose 6-phosphate alone (due to the presence of trace metals in the incubation buffer) stimulated very slightly hydroperoxide production, but the effect was much greater when Cu^{2+} was added to the incubation mixture. On the contrary, addition of DTPA completely prevented H_2O_2 production. The incubation of the enzyme with 5 mM glucose 6-phosphate did not determine any formation of hydrogen peroxide (data not shown). The slight decrease in Na^+/K^+ ATPase activity observed with copper alone is indicative of a direct but weaker effect of the metal on the cation pump and of a lower generation of oxidizing species. The addition of 100 U/ml catalase, which converts H_2O_2 to O_2 and H_2O, to the samples prior to the FOX reagent, reduced the color yield of FOX by 93% (see again Table 3). This indicates the formation of small amounts of water-soluble hydroperoxides, which could alter the boundary lipid environment of the enzyme.[7,9]

The concentrations of hydrogen peroxide produced, though low in absolute terms, are in the range expected to generate hydroxylating agents which may be responsible for protein oxidation.[8,11,25] Furthermore, H_2O_2 has been already reported to inactivate Na^+/K^+ ATPase[26] Recently, Rabini *et al.*[27] reported of a human plasma fraction inhibiting Na^+/K^+ ATPase identified as lysophosphatidylcholine (LPC). They suggested that the increased LPC plasma concentrations in diabetic patients might be caused by an increased oxidative degradation of plasma lipids. Since H_2O_2 is able to promote lipid peroxidation, a relationship between the increased hydroperoxides production and the decreased Na^+/K^+ ATPase activity found in diabetic patients may be suggested.

Eosin Y binds with a high affinity to the E_1 conformation of the Na^+/K^+ ATPase resulting in an increase in fluorescence, but with a low affinity to the E_2 conformation that we used for our experiments.[21–23] Eosin Y can thus be used as an extrinsic fluorescence probe to distinguish between different conformational states of the enzyme. Furthermore, eosin Y seems to bind to the ATP site of the enzyme.[21]

The incubation with glucose 6-phosphate or glucose 6-phosphate plus Cu^{2+} induced neither an increase in the extrinsic fluorescence nor the appearance of a shoulder on the excitation curve around 490 nm, which is characteristic of the enzyme in the E_1 conformation (data not shown). This would indicate that the production of hydrogen peroxide, observed in the early phases of the experimental glycation of Na^+/K^+ ATPase, induced no conformational modification and no alteration in the ATP binding site of the enzyme. This latter data is even more relevant since the ATP binding site contains a lysine residue which can be involved in the glycation process.[3,16]

The decrease in Na^+/K^+ ATPase activity, induced by H_2O_2 and hydroxylating agents following incubation with glucose 6-phosphate or glucose 6-phosphate plus Cu^{2+}, can not be ascribed

to a modification of tryptophan and/or tyrosine residues of the enzyme, which are usually very sensitive to oxidative attacks, since there were no statistically significant differences in intrinsic fluorescence data (Table 4). Furthermore, formation of glycofluorophores or AGEs can be excluded as well, since there was not any development of non-tryptophan fluorescence, probably due to the short time of incubation (see again Table 4).

Magnesium is a necessary cofactor for Na^+/K^+ ATPase activity and for many of the partial reactions of the cation pump.[28] Since preincubation with $MgCl_2$ did not prevent the dramatic decrease of enzymatic activity following the incubation with glucose 6-phosphate and copper or copper alone (Table 5), it would appear that the magnesium binding sites of the enzyme are not modified by H_2O_2 or hydroxylating agents. Since both $CuCl_2$ and $CuSO_4$ provoked the same degree of enzyme inactivation, any 'salt effect' of chloride and sulphate ions in the inactivation process can be excluded, remarking the important role of copper in the experimental glycation of brain Na^+/K^+ ATPase.

In conclusion, if experimental glycation is an adequate model of tissue protein damage occurring in diabetes mellitus,[29–31] our results suggest that an oxidative component may contribute to the glucose-mediated macromolecular damage and that such an effect may be important, together with a decompartmentalization of transition metals, in alterations frequently ascribed to glycation per se. The presence of glycoxidation products in lens proteins and tissue collagens and urine[5,29,32,33] indicates that oxidation accompanies glycation *in vivo* and that both oxidative and non-oxidative reactions can contribute to the chemical modification of tissue proteins by glucose.

Na^+/K^+ ATPase is a pertinent example of an enzyme, which is held to be functionally and structurally modified in diabetes[27] and altered by exposure to hyperglycemia, as shown by recent *in vivo* studies.[34,35] In our study, glucose 6-phosphate was used for the nonenzymatic glycation of Na^+/K^+ ATPase, since it is specific for amino groups.[3] In fact, protonation and deprotonation of certain amino groups are involved in the functional modulation of Na^+/K^+ ATPase.[21–23] Therefore, glucose 6-phosphate could lead to the *in vivo* modification of Na^+/K^+ ATPase, which, in turn, results in an altered function of this critical cation transport system.

Our data suggest that reduction in enzyme activity could be due to an oxidative modification. In fact, DTPA, chelating transition metal ions, does not interfere with glucose attachment to the enzyme molecule, but can block the oxidative damages induced by H_2O_2.

There is increasing debate about the role of protein glycation and free radical production in the development of diabetic complications, such as nephropathy, retinopathy and neuropathy.[1,25,29] Our study suggests, for the first time, that a critical metal-catalyzed oxidative step, with the production of hydroperoxides, plays a major role in the inactivation of Na^+/K^+ ATPase during the early phases of the experimental glycation. This oxidative reaction can be relevant to the development of experimental diabetic neuropathy since a reduction of the cation pump is postulated to play a critical role in the pathogenesis of diabetic neuropathy in animal model[12,13] and hydroperoxides and copper appear to be elevated especially in diabetic patients with complications.[5,7,11,25,29,36,37]

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References

1. V.M. Monnier (1990) Nonenzymatic glycosylation, the Maillard reaction and the aging process. *Journal of Gerontology- Biological Science*, **45**, B105–B111.
2. M.H. Garner and A. Spector (1985) Glucose-6-phosphate modification of bovine renal Na^+/K^+ ATPase: a model for changes occurring in the human renal medulla in diabetes. *Biochemical and Biophysical Research Communications*, **131**, 1206–1211.
3. M.H. Garner, A. Bahador and G. Sachs (1990) Nonenzymatic glycation of Na,K-ATPase. *Journal of Biological Chemistry*, **265**, 15 058–15 066.
4. H.F. Bunn and P.J. Higgins (1981) Reaction of monosaccharides with proteins: possible evolutionary significance. *Science*, **213**, 222–224.

5. V.M. Monnier and J.W. Baynes (1989) *The Maillard Reaction in Aging, Diabetes, and Nutrition*. Alan R. Liss Press, New York.
6. D.G. Dyer, J.A. Blackledge, B.M. Katz, C.J. Hull, H.D. Adkisson, S.R. Thorpe, T.J. Lyons and J.W. Baynes (1991) The Maillard reaction in vivo. *Zeitschrift fur Ernahrungswissenschaft*, **30**, 29–45.
7. S.P. Wolff, Z.Y. Jiang and J.V. Hunt (1991) Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radical Biology and Medicine*, **10**, 339–352.
8. Z.Y. Jiang, A.C.S. Wollard and S.P. Wolff (1990) Hydrogen peroxide production during experimental protein glycation. *FEBS Letters*, **268**, 69–71.
9. Z.Y. Jiang, J.V. Hunt and S.P. Wolff (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Analytical Biochemistry*, **202**, 384–389.
10. S.P. Wolff and R.T. Dean (1987) Glucose autoxidation and protein modification. *Biochemical Journal*, **245**, 243–250.
11. J.V. Hunt, R.T. Dean and S.P. Wolff (1988) Hydroxyl radical production and autoxidative glycosylation: glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *Biochemical Journal*, **256**, 205–212.
12. D.A. Green and S.A. Lattimer (1983) Na^+/K^+ ATPase defect in diabetic rat peripheral nerve: correction by myo-inositol administration. *Journal of Clinical Investigation*, **72**, 1050–1063.
13. P.K. Das, G.M. Bray, A.J. Aguayo and M. Rasminsky (1976) Diminished ouabain-sensitive, sodium-potassium ATPase activity in sciatic nerves of rats with streptozotocin-induced diabetes. *Experimental Neurology*, **53**, 285–288.
14. T. Matsuoka, M. Kato and K.J. Kako (1990) Effect of oxidants on Na^+/K^+ ATPase and its reversal. *Basic Research in Cardiology*, **85**, 330–341.
15. K. Kako, M. Kato, T. Matsuoka and A. Mustapha (1988) Depression of membrane-bound Na^+/K^+ ATPase activity induced by free radicals and by ischemia of kidney. *American Journal of Physiology*, **254**, C330–C337.
16. M.H. Garner and A. Spector (1987) Direct stimulation of Na^+/K^+ ATPase and its glucosylated derivative by aldose reductase inhibitor. *Diabetes*, **36**, 716–720.
17. A.K. Mallia, G.T. Hermanson, R.I. Krohn, E.K. Fujimoto and P.K. Smith (1981) Preparation and use of a boronic acid affinity support for separation and quantitation of glycosylated hemoglobins. *Analytical Letters*, **14**, 649–661.
18. D.K. Klenk, G.T. Hermanson, R.I. Krohn, E.K. Fujimoto, A.K. Mallia, P.K. Smith, J.D. England, H.M. Wiedmeyer, R.R. Little and D.E. Goldstein (1982) Determination of glycosylated hemoglobin by affinity chromatography: comparison with colorimetric and ion-exchange methods, and effects of common interferences. *Clinical Chemistry*, **28**, 2088–2094.
19. M.M. Bradford (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.
20. J.G. Nørby (1988) Coupled assay of Na^+/K^+ ATPase activity. *Methods in Enzymology*, **156**, 116–119.
21. J.C. Skou and M. Esmann (1988) Eosin as a fluorescence probe for measurement of conformational states of Na^+/K^+ ATPase. *Methods in Enzymology*, **156**, 278–281.
22. J.C. Skou (1982) The effect of pH, of ATP and of modification with pyridoxal 5-phosphate on the conformational transition between the Na^+-form and the K^+-form of the (Na^+ + K^+)-ATPase. *Biochimica et Biophysica Acta*, **688**, 369–380.
23. J.C. Skou and M. Esmann (1980) Effect of ATP and protons on the Na:K selectivity of the (Na^+ + K^+)-ATPase studied by ligand effects on intrinsic and extrinsic fluorescence. *Biochimica et Biophysica Acta*, **601**, 386–402.
24. J.V. Hunt, M.A. Bottoms and M.J. Mitchinson (1993) Oxidative alterations in the experimental glycation model of diabetes mellitus are due to protein-glucose adduct oxidation. *Biochemical Journal*, **291**, 529–535.
25. S.P. Wolff (1987) The potential role of oxidative stress in diabetes and its complications: novel implications for theory and therapy. In *Diabetic complications: scientific and clinical aspects* (ed. M.J.C. Crabbe), Churchill Livingstone, Edinburgh, pp. 167–220.
26. M.H. Garner, W.H. Garner and A. Spector (1984) Kinetic cooperativity change after H_2O_2 modification of (Na,K)-ATPase. *Journal of Biological Chemistry*, **259**, 7712–7718.
27. R.A. Rabini, R. Galassi, P. Fumelli, N. Dousset, M.L. Solera, P. Valdiguie, G. Curatola, G. Ferretti, M. Taus and L. Mazzanti (1994) Reduced Na^+-K^+ ATPase activity and plasma lysophosphatidylcholine concentrations in diabetic patients. *Diabetes*, **43**, 915–919.
28. S.L. Bonting, H.G.P. Swarts, W.H.M. Peters, F.M.A.H. Schurmans Stekhoven, J.J.H.H. De Pont (1983) Magnesium-induced conformational changes in Na,K-ATPase. In *Structure, Mechanism, and Function of the Na/K Pump* (eds. J.F. Hoffman and B. Forbush III), Academic Press, London and New York, pp. 403–424.
29. J.W. Baynes (1991) Role of oxidative stress in development of complications in diabetes. *Diabetes*, **40**, 405–412.
30. M.X. Fu, K.J. Wells-Knecht, J.A. Blackledge, T.J. Lyons, S.R. Thorpe and J.W. Baynes (1994) Glycation, glycoxidation, and cross-linking of collagen by glucose. *Diabetes*, **43**, 676–683.
31. K.V. Chace, R. Carubelli and R.E. Nordquist (1991) The role of nonenzymatic glycosylation, transition metals, and free radicals in the formation of collagen aggregates. *Archives of Biochemistry and Biophysics*, **288**, 473–480.
32. S.K. Grandhee and V.M. Monnier (1989) Mechanisms of formation of the Maillard protein cross-link pentosidine: glucose, fructose, and ascorbate as pentosidine precursors. *Journal of Biological Chemistry*, **264**, 21 592–21 602.
33. J.A. Dunn, J.S. Patrick, S.R. Thorpe and J.W. Baynes (1989) Oxidation of glycated proteins: age-dependent accumulation of N^{ε}-(carboxymethyl)lysine in lens proteins. *Biochemical Journal*, **28**, 9464–9468.
34. T.S. Lee, L.C. MacGregor, S.J. Fluharty and G.L. King (1989) Differential regulation of protein kinase C and (Na,K)-adenosine triphosphatase activities by elevated glucose levels in retinal capillary endothelial cells. *Journal of Clinical Investigation*, **83**, 90–94.
35. S. Gupta, I. Sussman, C.S. McArthur, K. Tornheim, R.A. Cohen and N.B. Ruderman (1992) Endothelium-dependent inhibition of Na^+-K^+ ATPase activity in rabbit aorta by hyperglycemia. *Journal of Clinical Investigation*, **90**, 727–732.
36. A.F. Jones, J.W. Winkles, P.E. Jennings, C.M. Florkowski, J. Lunec and A.H. Barnett (1988) Serum antioxidant activity in diabetes mellitus. *Diabetes Research*, **7**, 89–92.
37. R.M. Walter, J.Y. Uri-Hare, K.L. Olin, M.H. Oster, B.D. Anawalt, J.W. Critchfield and C.L. Keen (1991) Copper, zinc, magnesium status and complication of diabetes mellitus. *Diabetes Care*, **14**, 1050–1056.