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SUPEROXIDE RADICAL GENERATION BY AMADORI COMPOUNDS

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Several D-sugars were incubated with L-lysine or with L-arginine for 10 days. The resulting compounds are able to reduce nitrobluetetrazolium (NBT). This is prevented by superoxide dismutase (SOD), indicating that the superoxide radical is generated by the resulting Amadori compounds.

The formation of superoxide radical *in vivo*, as a result of nonenzymatic glycosylation of proteins, may be considered to be a contributory factor to the appearance of chronic complications of diabetes.

KEY WORDS: Superoxide radicals, glycosylation, Amadori compounds, diabetes.

RESULTS

Compounds resulting from the nonenzymatic glycosylation of proteins have been studied for many years.[1] Reducing sugars react quickly with compounds containing a primary amine group, forming glycosylamines.[2] Products resulting from reactions between sugars and aminoacids depend on the molar ratio of these substances.[3] From these different possible reactions, coloured products of high molecular weight – the melanoidins – are obtained either slowly at physiological temperature or rapidly at 80°C.[4]

The melanoidins together with early uncoloured compounds are responsible in part for the late complications of diabetes.[5] Also the normal aging process seems to be related to the accumulation of compounds which result from nonenzymatic glycosylation of proteins and of other molecules such as nucleic acids.[6]

The binding of the amine groups of proteins to carbonyl groups of sugars originates a reversible Schiff's base which undergoes a rearrangement forming Amadori compounds.[7] Both the structure and the function of the protein undergo alterations.[5,6] D-isoglucosamine (1-amine-deoxi-D-fructose) is typical of the compounds produced in the amine-carbonyl reactions. It may be synthesised through the reaction between glucose and lysine, or it may be generated *in vivo* from the reaction of the carbonyl group of glucose with the ε-amine group of lysine.[8]

It has been demonstrated that NBT reduction by fructosamine in glycolsated proteins is inhibited by SOD, and this suggests that Amadori compounds produce the superoxide radical.[9]

The purpose of the present work is to investigate if glycosylamines formed by Amadori rearrangement present a similar behaviour in relation to NBT and what is the effect of SOD.

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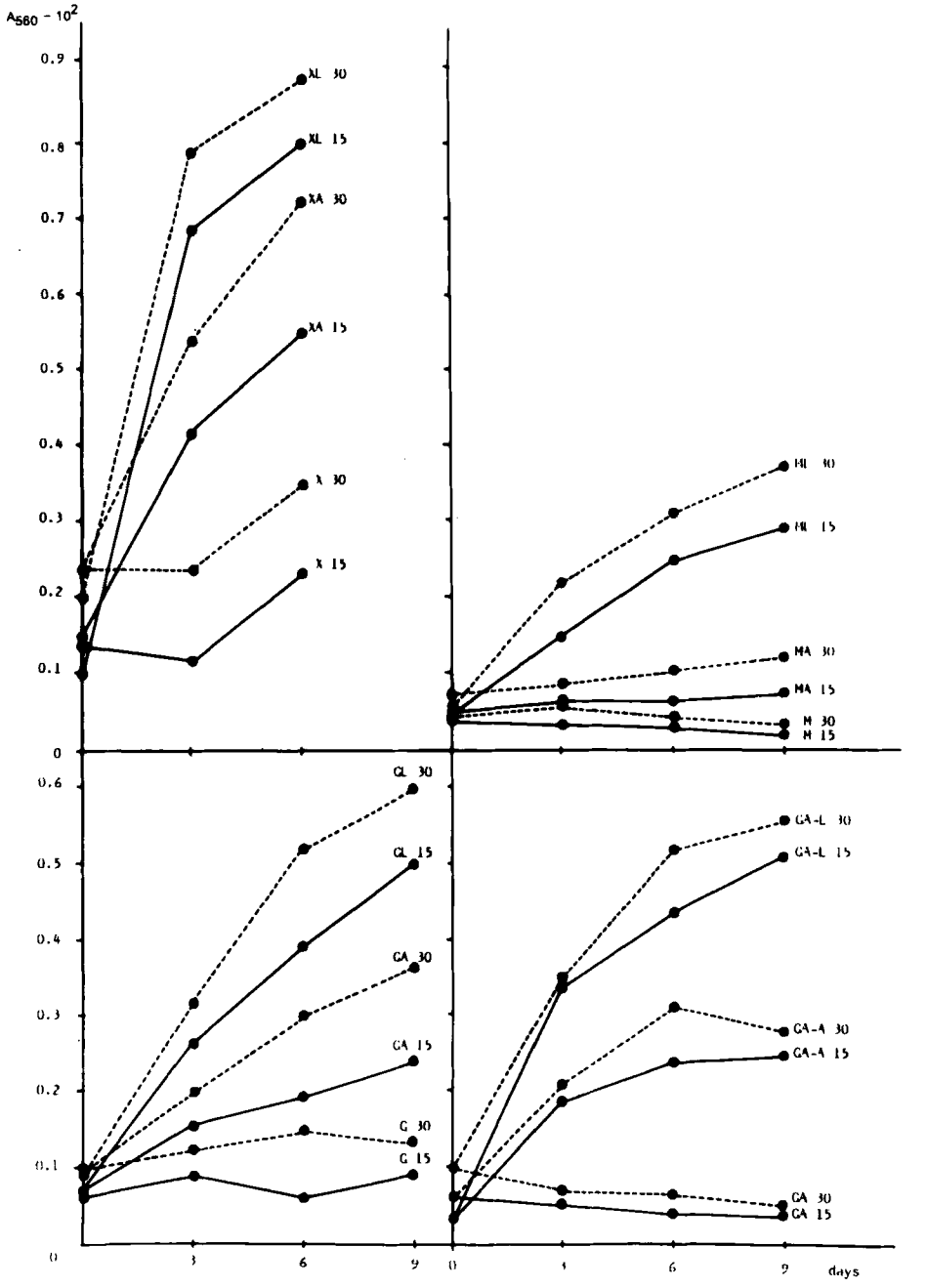

FIGURE 1 D-glucose (G), D-galactose (GA), D-mannose (M) and D-xylose (X) incubated with L-lysine (L) or with L-arginine (A). At days 0 (1 hour), 3, 6 and 9 samples of 100 μl of the incubation mixtures are added to 800 μl NBT + 100 μl water. ΔA_{560} nm are recorded after 15 and after 30 min. Points represent means of 4 similar experiments.

MATERIAL AND METHODS

Reagents of the highest purity were purchased and used without further purification. D-sugars, L-aminoacids, NBT and SOD were obtained from Sigma, Sodium carbonate, sodium mono and diphosphate from Merck.

Aminoacids and sugars were dissolved in sodium phosphate buffer 0.05 M, pH = 7.4 to concentration of 40 mM. Two ml of sugar and 2 ml of aminoacid solutions were mixed, decreasing the final concentration of both to 20 mM.

Hibitane was added to a final concentration of 1/2000, to avoid bacterial contamination. The incubations lasted up to 10 days at 37°C in a shaking water bath, except with L-xylose which had to be discontinued after 6 days because a precipitate appeared due to excessive reduction of NBT.

At days 0 (1 bour), 3, 6 and 9, 0.1 ml of the mixture was withdrawn and this added to 0.8 ml of NBT solution (0.25 mM in carbonate buffer 0.1 M, pH = 10.8). A final volume of 1 ml was completed with 0.1 ml buffer. This mixture was incubated for 15 or 30 minutes and ΔA_{560} determined against a blank containing 0.8 ml NBT and 0.2 ml phosphate buffer.

In the 10th day a similar experiment was made with and without SOD, 5000/ml (0.8 ml NBT + 0.1 ml incubating mixture + 0.1 ml SOD in buffer). All experiments were carried out in quadruplicate.

RESULTS

Figure 1 shows the results obtained in the first 9 days. When the sugars were incubated alone, the observed reactivity with NBT is small, except for xylose at the 6th day. Incubations of aminoacids alone do not bring any changes (data not presented).

The mixtures of sugars and aminoacids however show remarkable changes. L-xylose is the sugar which causes highest reduction, followed by L-glucose and L-galactose, which are similar. L-mannose induced a smaller NBT reactivity. L-lysine is more active than L-arginine in inducing NBT reduction.

The experiments with SOD at the 10th day (6th day for L-xylose) are presented in Table 1. A significanty decrease in ΔA^{560} is observed in the presence of SOD, even in the cases in which sugars were incubated alone.

DISCUSSION

The occurrence of Amadori rearrangements is easily detected by its reducing properties.[3] The mechanism of the reaction of the formation of formazan has been studied by several investigators: the salts of tetrazolium compounds undergo reduction processes and are transformed in formazan, of violet colour.[10]

At the pH used, the superoxide radical is essentially a reductant. Its fomation results possibly from the rearrangement of the Amadori compound with formation of a sugar free-radical and of superoxide, in the presence of dioxygen. SOD inhibits the formation of formazan by transforming O_2^- into H_2O_2. The fact that autoxidation of sugars generates O_2^- together with sugar free-radicals has been described previously.[11,13]

TABLE I

Effect of SOD on the reduction of NBT by incubation mixtures, containing D-sugars and L-aminoacids in equal concentrations (20 mM). Incubation time 10 days, except for L-xylose (6 days)

Incubation system	With SOD		Without SOD		t-test		% inhibition by SOD	
	15 min	30 min	15 min	30 min	15 min	30 min	15 min	30 min
D-glucose	0.0335 ± 0.0119	0.0651 ± 0.0086	0.0651 ± 0.0086	0.1613 ± 0.0139	t = 4.3040 p < 0.01	t = 11.7700 p < 0.001	49	60
D-glucose + L-lysine	0.1627 ± 0.0094	0.2398 ± 0.0536	0.5410 ± 0.0955	0.6855 ± 0.0577	t = 7.8844 p < 0.001	t = 11.3187 p < 0.001	70	65
D-glucose + L-arginine	0.1068 ± 0.0057	0.1647 ± 0.0304	0.2052 ± 0.0448	0.4058 ± 0.0244	t = 23.8800 p < 0.001	t = 12.3715 p < 0.001	48	55
D-galactose	0.0158 ± 0.0048	0.0245 ± 0.0038	0.0578 ± 0.0033	0.0665 ± 0.0006	t = 4.444 p < 0.01	t = 21.8348 p < 0.001	73	63
D-galactose + L-lysine	0.1332 ± 0.0063	0.1453 ± 0.0036	0.5705 ± 0.0095	0.6115 ± 0.0075	t = 76.7164 p < 0.001	t = 112.0770 p < 0.001	77	76
D-galactose + L-arginine	0.0675 ± 0.0037	0.0675 ± 0.0017	0.2578 ± 0.0122	0.2985 ± 0.0056	t = 29.8530 p < 0.001	t = 78.9426 p < 0.001	74	77
D-mannose	0.0025 ± 0.0001	0.0115 ± 0.0006	0.0180 ± 0.0020	0.0365 ± 0.0019	t = 15.4806 p < 0.001	t = 25.0942 p < 0.001	86	68
D-mannose + L-lysine	0.1022 ± 0.0033	0.1232 ± 0.0025	0.3922 ± 0.0021	0.5083 ± 0.0057	t = 148.2479 p < 0.001	t = 123.7438 p < 0.001	74	76
D-mannose + L-arginine	0.0190 ± 0.0008	0.0278 ± 0.0030	0.0785 ± 0.0084	0.1338 ± 0.0093	t = 14.1028 p < 0.001	t = 21.6948 p < 0.001	76	79
D-xylose	0.0710 ± 0.0041	0.1300 ± 0.0067	0.1728 ± 0.0026	0.3483 ± 0.0178	t = 41.9370 p < 0.001	t = 5.5911 p < 0.001	59	63
D-xylose + L-lysine	0.2583 ± 0.0373	0.3080 ± 0.0313	0.7777 ± 0.6790	0.9048 ± 0.0396	t = 13.4064 p < 0.001	t = 23.6468 p < 0.001	67	66
D-xylose + L-arginine	0.1748 ± 0.0179	0.2648 ± 0.0100	0.5488 ± 0.0189	0.7408 ± 0.0470	t = 28.7107 p < 0.001	t = 19.8097 p < 0.001	68	64

The complications which accompany long term diabetes are essentially attributed to two causes both related to hyperglycemia. These are activation of the polyol pathway and nonenzymatic protein glycosylation.[5,12] The present investigation suggests another possible cause. This is the continuous formation of oxygen radicals by Amadori compounds. It would therefore be important to demonstrate that the SOD-inhibitable reduction of NBT correlates with the formation of the Amadori adduct, determined by an independent measure such as incorporation of radiolabelled sugars onto the aminoacids.

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