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Letter to the Editor

Aspirin inhibits the formation of pentosidine, a cross-linking advanced glycation end product, in collagen

Abstract

Aspirin showed an inhibitory effect on the formation of pentosidine, a cross-linking advanced glycation endproduct, in collagen incubated with glucose *in vitro*. IC_{50} was evaluated at 10 mmol/l. Aspirin might act by metallic ion chelating (as did EDTA and DTPA) and by oxygen radical scavenging. Since aspirin was reported to inhibit retinopathy in diabetic dogs, it could act partly by inhibiting advanced glycation endproduct accumulation in long-lived proteins like collagens.

Keywords: Aspirin; Salicylates; Pentosidine; Collagens; Advanced glycation endproducts (AGEs)

To the Editor,

Advanced glycation or glycoxidation of proteins, particularly long-lived collagens, appears to contribute to the development of microvascular complications in diabetes. Pentosidine is a specific advanced glycation endproduct (AGE), cross-linking peptidic chains and modifying the chemical properties of proteins. Skin collagen pentosidine levels, adjusted for age, have been shown to correlate with the severity of complications in type 1 diabetic patients; more recently, when adjusted for age and diabetes duration, they were significantly associated with nephropathy and neuropathy [1]. Aspirin has been reported to have beneficial effects on hypertension and vascular oxidative stress in chronically glucose-fed rats and on retinopathy in diabetics dogs [2]. Therefore it was interesting to examine the effect of aspirin on pentosidine formation in collagen incubated with high glucose. The effect of aspirin was compared with that of two chelators, DTPA and EDTA, since chelating activity has been implicated in the mechanism of action of various glycoxidation inhibitors.

Insoluble collagen from bovine Achilles tendon (6 mg/ml, Sigma, St. Louis, MO, USA) was incubated in 200 mmol/l sodium phosphate buffer, pH 7.4, with or without 250 mmol/l glucose, in the presence or absence of aspirin (Bottu-Theraplix, Paris, France) or DTPA (Acros, Geel, Belgium), or EDTA (Sigma), for 28 days at 37 °C, under air in 5 ml stoppered glass tubes, with 10 μl toluene per 1 ml final volume, in quadruplicates. Air and toluene were renewed weekly. Final pH was adjusted to 7.4 if necessary. Quercetin (Sigma, introduced in 20 μl ethanol) and pyridoxamine (Merck, Darmstadt, Germany) were used as reference standards. After incubation, collagen was washed, lyophilized and submitted to acid hydrolysis under vacuum. Pentosidine (P) was measured by HPLC, using a standard given by Vincent Monnier (Case Western University, Cleveland) [1]; hydroxyproline (Hyp) was determined colorimetrically. Results were expressed as means ± S.E.M. Statistical comparisons were performed by two-way analysis of variance. Dose–response curve was obtained using Graph Pad Prism® 3.02 software.

In the presence of glucose alone, P/Hyp = 134.8 ± 9.2 pmol P/μmol Hyp (versus 8.8 ± 1.4 in the absence of glucose, $p < 0.001$). At 40 mmol/l, aspirin reduced P/Hyp to 52.5 ± 17.4 pmol/μmol (34.7% of glucose control specific P/Hyp, $p < 0.01$). It was inactive at 1 mmol/l (128.0 ± 16.6 pmol/μmol; 94.6%). DTPA very markedly reduced P/Hyp to 17.3 ± 4.2 pmol/μmol (6.7%, $p < 0.001$) at 40 mmol/l and to 14.7 ± 0.6 pmol/μmol (5%, $p < 0.001$) at 10 mmol/l. EDTA lowered P/Hyp to 40.2 ± 4.6 pmol/μmol (25%, $p < 0.001$) at 40 mmol/l and to 51.9 ± 15.5 pmol/μmol (35%, $p < 0.001$) at 10 mmol/l. As expected, pyridoxamine reduced P/Hyp to the value of the control without glucose both at 40 and 90 mmol/l; quercetin reduced

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P/Hyp to 47.1 ± 10.6 pmol/μmol (30.4%, $p < 0.001$) at 250 μmol/l and to 70.9 ± 15.9 pmol/μmol (48.6%, $p < 0.01$) at 25 μmol/l.

Two mechanisms may be involved in the aspirin effect on glycoxidation, oxygen radical scavenging [3] and metal ion chelation: at 0.1 mmol/l concentration, aspirin, EDTA and DTPA significantly reduced Fe^{2+}-ferrozine formation to 77%, 12.1% and 10.9% of control, respectively [4]. In our experimental model, the importance of trace metal ions is confirmed by the effects of DTPA and EDTA.

Effects of aspirin on glycoxidation *in vitro* had been reported in symposium proceedings – not included in PubMed internet data library – that we discovered *a posteriori* [5]: aspirin reduced pentosidine formation in collagen incubated in 250 mmol/l glucose for 21 days to 50%, 36% and 6% of control, respectively, at 10, 25 and 100 mmol/l concentrations. Salicylate reduced it to 29%, 14% and 4%, respectively. Our experimental conditions were very similar to those of Fu et al., except that they measured pentosidine in the pepsin-solubilized collagen fraction after incubating 9 mg collagen per ml glucose medium, whereas we used the total insoluble collagen after incubating 6 mg collagen per ml glucose medium. If we might gather our results and those of Fu et al., they would fit together nicely in a complementary way resulting in a unique completed sigmoid dose–response curve with a 50% inhibition concentration IC_{50} of 10 mmol/l.

Aspirin (20 mmol/l) was also reported to decrease total AGE content (as measured by competitive ELISA, using anti-AGE-albumin antibodies) and pepsin resistance of collagen, which had been incubated with glucose [6].

No effect of aspirin at 10, 25 and 100 mmol/l concentrations on Amadori product formation was observed in collagen when incubated in 250 mmol/l glucose for 21 days [5]. Previous studies had reported inhibition of Amadori product formation by acetylation of amino groups. But these studies were compromised by several factors: high concentrations of aspirin; poor pH control; measurements of glycation by radiochemical assays which do not distinguish glycation from glycoxidation; measurements of glycation by relatively non-specific assays, such as thiobarbituric acid assay; specific binding of aspirin to the protein under study [5].

Normalization of tendon collagen thermal rupture time in streptozotocin-diabetic rats treated by aspirin 240 mg/kg body weight during 4 weeks was described (AGEs were not directly determined) [7]. Reduction of diabetic retinopathy (particularly retinal hemorrhages and acellular capillaries) was reported in 5-year-alloxan-diabetic dogs treated by aspirin (20 mg/kg) [2]. However no effect on the accumulation of pentosidine or total AGEs in aorta or tail tendon collagen was observed neither in aspirin- nor in aminoguanidine-treated animals.

Twenty-four *type 2-diabetic patients* received either aspirin (100 mg/day) or placebo during 1 year [8]. Subjects receiving aspirin had decreased levels of skin pentosidine ($p < 0.05$). No effects of aspirin on clinical scores of retinopathy, nephropathy or neuropathy were observed.

In vitro a freshly prepared aspirin solution at pH 7.4 at 17 °C can be expected to exhibit 10% hydrolysis into salicylate after 1 day, corresponding to a half-life of 6 days. *In human* after oral aspirin ingestion, only 68% of the dose reaches the systemic circulation as aspirin where it is converted into salicylate by nonspecific esterases; the half-life of aspirin is about 20 min; that of salicylate is much longer, 3–4.5 h [9]. At therapeutic concentrations for optimal anti-inflammatory effect, 1.1–2.2 mmol/l according to [9], salicylate is bound to albumin at 80–90%. Free salicylate can be filtered by the kidney glomerulus and secreted by the tubule in order to be excreted in urine where it is almost entirely unbound since proteinuria is minimal. Salicylate can also be conjugated with glycine or glucuronic acid or oxidized into gentisic acid which are eliminated in urine.

Therapeutic plasma salicylate concentration of 2.2 mmol/l corresponds to 19% inhibition of pentosidine formation on the aspirin dose–response curve and about 34% inhibition on the salicylate dose–response curve deducted from the results of Fu et al. (which shows an IC_{50} of 4 mmol/l). In urine unconjugated salicylate concentration can reach 6.2 mmol/l for a plasma level of 2.2 mmol/l [10]: then inhibition of pentosidine formation in the surrounding kidney proteins may reach 33% if we consider the aspirin dose–response curve and about 60% inhibition if we consider the salicylate dose–response curve.

Higher local salicylate concentrations can be obtained in skin after triethanolamine salicylate topical application. In diabetic patients this may be interesting for inhibiting dermal collagen induration in limited areas [11]. In canine studies, salicylate concentration reached indeed 51 mmol/kg skin tissue, 1 h after 10 g of 10% salicylate ointment application, whereas serum level was limited to 4 μmol/l [12].

We must be aware that the composition of our medium *in vitro* differs from that in plasma which is in contact with capillary basement membrane collagens.