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Inhibition of Heat-induced Denaturation of Albumin by Nonsteroidal Antiinflammatory Drugs (NSAIDs): Pharmacological Implications

Luciano Saso, Giovanni Valentini, Maria Luisa Casini, Eleonora Grippa, Maria Teresa Gatto, Maria Grazia Leone, and Bruno Silvestrini

Department of Pharmacology of Natural Substances and General Physiology, University of Rome "La Sapienza", Rome, Italy

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The activity of nonsteroidal antiinflammatory drugs (NSAIDs) in rheumatoid arthritis is not only due to the inhibition of the production of prostaglandins, which can even have beneficial immunosuppressive effects in chronic inflammatory processes. Since we speculated that these drugs could also act by protecting endogenous proteins against denaturation, we evaluated their effect on heat-induced denaturation human serum albumin (HSA) in comparison with several fatty acids which are known to be potent stabilizers of this protein. By the Mizushimas assay and a recently developed HPLC assay, we observed that NSAIDs were slightly less active [EC_{50}~10^{-5}-10^{-4} M] than FA and that the HPLC method was less sensitive but more selective than the turbidimetric assay, i.e. it was capable of distinguishing true antiaggregant agents like FA and NSAIDs from substances capable of inhibiting the precipitation of denatured protein aggregates. In conclusion, this survey could be useful for the development of more effective agents in protein condensation diseases like rheumatic disorders, cataract and Alzheimers disease.

Key words: Anti-rheumatic agents, Protein denaturation, Nonsteroidal antiinflammatory drugs, Fatty acids, Protein condensation diseases

INTRODUCTION

The activity of nonsteroidal antiinflammatory drugs (NSAIDs) in rheumatoid arthritis and other rheumatic diseases does not seem to be only due to the inhibition of the production of endogenous prostaglandins, which could be affected at much lower doses than those required in these conditions (Insel, 1996). Besides, this action could not be completely beneficial since some of these autacoids possess significant immunosuppressive properties in chronic inflammatory diseases (Insel,1996). However, up to date, only a few alternative hypothesis have been proposed (Goodwin, 1984a; Goodwin, 1984b; Abramson and Weissmann, 1989; Abramson, 1990; Abramson, 1991; Diaz-Gonzalez and Sanchez-Madrid, 1998)

The lack of knowledge in this area is partly due to the brilliant discovery of the inhibition of cyclo-oxygenase (COX) by aspirin-like drugs (Vane, 1971) which induced many scientists to abandon other research lines. One of these (Mizushima, 1964; Mizushima and Suzuki, 1965) was particularly convincing because it was based on the parallelism between two physio-pathological phenomena, i.e. inflammation and protein denaturation: since both of them could be caused by the same agents (heat, radiations, organic solvents, etc.) and NSAIDs could protect proteins against denaturation *in vitro*, their antiinflammatory activity could be due to their antidenaturant activity. However, only a few obstinate researchers kept working on this idea in the new prostaglandin era, convinced that the effect on COX was distinct from the antidenaturant one: the former could be of therapeutic value in acute inflammatory conditions, while the latter could be more important in chronic degenerative disorders (Silvestrini, 1969 and 1987). Several chemical

Correspondence to: Dr. Luciano Saso, Department of Pharmacology of Natural Substances and General Physiology, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy
E-mail: luciano.saso@uniroma1.it

analogues were synthesized in the attempt of separating the two activities: among these, benzydamine appeared very effective in acute but not in chronic inflammatory models (Bertelli and Silvestrini,1985; Cioli *et al*, 1985) while bendazac (Silvestrini *et al.*, 1969; Silvestrini *et al.*, 1970; Silvestrini, 1987) and, more recently, bindarit (Cioli *et al.*, 1992) showed a completely opposite pharmacological profile: it is of note that the latter was effective in the secondary phase of Freunds adjuvant arthritis, an experimental model of rheumatoid arthritis without having any activity on COX or acute inflammatory models (Cioli *et al.*, 1992). Strictly related to these observations is the recent report that fatty acids (FA), supplemented in high doses in chronic rheumatic disorders (van der Tempel, 1990; Kremer, 1996), could exert beneficial effects also by stabilizing endogenous proteins (Saso *et al.*, 1999).

Thus, almost forty years after Mizushimas work on NSAIDs (Mizushima, 1964), we decided to reevaluate their effect on heat-induced denaturation of albumin *in vitro*, using more modern techniques, i.e. a modified Mizushimas test (Saso et *al.*, 1998; Saso et *al.*, 1999) and a gel permeation high performance liquid chromatographic (HPLC) assay, capable of distinguishing between true antiaggregant substances and denaturing solubilizing agents (Saso *et al.*, 1998). In addition, since FA are potent stabilizer of human serum albumin (HSA) (Saso *et al.*, 1999), we decided to test FA and NSAIDs on both the fatted (HSA_V) and the defatted (HSA_d) protein.

MATERIALS AND METHODS

Reagents

Normal human serum (NHS) was obtained from healthy volunteers. Sodium chloride (cat. N° S-9888), sodium phosphate (product N° S-9638), Tris (product N° T-1503) human serum albumin [96-99% pure, Cohns fraction V, product N°A1653 (HSA_V); and defatted, ≥ 96% pure, essentially fatty acid (FA) free, product N° A-1887 (HSA_d)], caprylic acid (product N° C-5038), capric acid (product N° C-1875), lauric acid (product N° L-4250), myristic acid (product N° M-3253), palmitic acid (product N° P-0500), stearic acid (product N° S-4751), oleic acid (product N° O-1008), linoleic acid (product N° L-1376), γ-linolenic acid (product N° L-2378), α-linolenic acid (product N° L-2376), arachidic acid (product N° A-3631), arachidonic acid (product N° A-3925), eicosapentaenoic acid (product N° E-2011), docosahexaenoic acid (product N° D-2534), diflunisal (product N° D-3281), salicylic acid (product N° S-0875), acetaminophen (product N° A-5000), indomethacin (product N° I-7378), sulindac (product N° S-8139), diclofenac (product N° D-6899), tolmetin (product N° T-6779), fenoprofen (product N° F-1517), flurbiprofen (product N° F8514), ibuprofen (product N° I-1892), ketoprofen (product N° K-1751), naproxen (product N° M-4015), meclofenamic acid (product N° M-4531), mefenamic acid (product N° M-4267), phenylbutazone (product N° P-8386), piroxicam (product N° P-5654), nabumetone (product N° N-6142), acrylamide (product N° A-4058) and sodium thiocyanate (cat. N° S-7757) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS, cat. N° 161-0301), bromophenol blue (cat. N° 161-0404) and other reagents for electrophoresis were from Bio-Rad (Richmond, CA, USA). Coomassie blue R-250 and G-250 were from United States Biochemical Co. (Cleveland, Ohio, USA). Bio-Rad (Richmond, CA, USA). High performance liquid chromatography (HPLC) solvents were from Lab-Scan Analytical Sciences (Dublin, Ireland).

Purification of albumin from normal human serum

Albumin was purified from NHS by Affi-gel blue affinity chromatography, according to (Cheng *et al*, 1988). Briefly, a 10 x 500 mm column (Econo-column®, Bio-Rad cat. N° 737-1052) was packed with 40 ml of Affi-gel blue (50-100 mesh, Bio-Rad cat. N° 153-7301) and equilibrated with buffer A (0.02 M Tris pH 7.4 at 22°C) at the flow rate of 6 ml/hr. Then, 10 ml of NHS, extensively dialyzed against buffer A using a dialysis membrane with cut-off 6000-8000 daltons (Spectra/Por®, cat. N° 132650, Spectrum, Houston, Texas, USA), were loaded onto the column at the same flow rate, and serum globulins (G) were collected until the absorbance of the eluate at 214 nm was low and stable. Then, HSA_{NHS} was eluted with 200 ml of buffer B (0.05 M Tris, pH 8.0 at 22°C, containing 0.2 M sodium thiocyanate). Finally, the two fractions were dialyzed against buffer A using an 8050 Amicon (Danvers, MA, USA) ultrafiltration unit, equipped with YM-10 membranes with cut-off 10000 daltons, and concentrated down to 10 ml. The protein content of both serum fractions was estimated by a Coomassie blue binding assay performed according to (Bradford, 1976) as modified by (Macart and Gerbaut, 1982).

The purity of HSA_{NHS} was verified by both poly-acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and reversed-phase high performance liquid chromatography (HPLC): (i) aliquots of about 20 µg of NHS, G, HSA_{NHS}, HSA_V and HSA_d were fractionated under reducing conditions (in the presence of 2-mercapto-ethanol) on a 7.5% *T* gel, according to (Laemmli, 1970), and the gel was stained with Coomassie blue R-250; (ii) about 100 µg of HSA_{NHS} were loaded onto a Vydac C_{18} reversed-phase column equilibrated at 1 ml/min with 20% of solvent A (5% acetonitrile, 95% water and 0.1% trifluoroacetic acid) in solvent B (95% acetonitrile, 5% water and 0.1% trifluoroacetic acid) and a gradient from 20 to 100% B was started, monitoring the eluate at 214 nm with a sensitivity of 1 A.U.F.S.

Heat-induced denaturation of albumins

HSA_{NHS}, HSA_V and HSA_d were dissolved at 0, 0.05, 0.1 and 0.2% in 0.066 M sodium phosphate (pH 5.3 at 22°C) and aliquots of 100 µl were pipetted in triplicate onto a 96-well microplate (Falcon 3911, Becton & Dickinson, Oxnard, CA, USA), the plate was covered with aluminum foil and incubated in oven at 70°C ± 0.1 °C for 30 min. The turbidity of the samples was determined at 595 nm, using an automatic microplate reader (model 3550, Bio-Rad, Hercules CA, USA).

HSA_V and HSA_d were dissolved at 0.2% (w/v) in 0.066 M sodium phosphate (pH 5.3 at 22°C) and heat-induced denaturation of aliquots of 100 µl was examined as described above at different temperatures in the range 40-95°C.

HSA_V and HSA_d were dissolved at 0.2% (w/v) in 0.02 M Tris (pH 8.0 at 22°C) and heat-induced aggregation was studied by HPLC as previously described (Saso *et al*, 1998). Briefly, aliquots of 0.5 ml were heated at different temperatures (40-95°C) for 5 min in a water bath, using borosilicate tubes in duplicate, immediately cooled for 10 min at 0°C, and aliquots of 50 µl (100 µg) were loaded onto a Bio-Sil TSK-250 column (7.5 × 300 mm i.d., 250 Å, permeation range 1000-300,000 daltons) equilibrated with TSK-buffer (0.05 M sodium phosphate pH 6.8 at 22 °C, containing 0.15 M NaCl) at the flow rate of 1 ml/min, monitoring the eluate at 280 nm with a sensitivity of 0.1 A.U.F.S. Then, the area under the peak of monomeric albumin (AUC) was measured using an electronic integrator (model 2221, Pharmacia-LKB) and the extent of aggregation was calculated using the following formula:

$$\text{aggregation (\%)} = 100 \cdot (1 - AUC_{T°C}/AUC_{22°C})$$

HSA_V was dissolved at 0.2% (w/v) in 0.02 M Tris (pH 8.0 at 22°C), aliquots of 0.5 ml were heated for 5 min in a water bath at 80°C as described above, in the presence or absence ($C_{80°C}$) of caprylic (10^{-3} M), capric (5×10^{-4} M) and lauric acid (5×10^{-4} M), and 20 µl (40 ug) of each sample were analyzed by PAGE on a 7.5% *T* under non-reducing conditions.

Effect of selected fatty acids and nonsteroidal antiinflammatory drugs on heat-induced denaturation of NHS and HSA.

Heat-induced denaturation of NHS, HSA_{NHS}, HSA_V and HSA_d, in the presence of selected FA and nonsteroidal antiinflammatory drugs (NSAIDs), was evaluated by both a modified version (Saso *et al*, 1998; Saso *et al*, 1999) of the classical Mizushima test (Mizushima, 1964) and a recently developed HPLC assay (Saso *et al*, 1998): (i) turbidimetric: briefly, FA were dissolved at their maximum solubility in ethanol and small volumes of these ethanolic solutions were added to either NHS (1:4 in sodium citrate, pH 5.3 at 22°C) or 0.2% (w/v) HSA; NSAIDs were dissolved at their maximal solubility in 0.066 M phosphate (pH 5.3 at 22°C), serially diluted and mixed 1:1 with 0.4% (w/v) HSA in the same buffer, and aliquots of 0.1 ml were pipetted in quadruplicate onto a 96-well microplate and the plates were heated for 30 min at 70°C as described above; the antidenaturant activity was calculated with the following formula:

$$a_{X,c} = [1 - (A_{X,\ 70°C} - A_{0,\ 22°C})/(A_{0,70°C} - A_{0,\ 22°C})] \cdot 100$$

where $A_{X,\ 70°C}$ is the absorbance at 595 nm of the sample heated in the presence of the substance X at the concentration c; $A_{0,\ 22°C}$ and $A_{0,\ 70°C}$ are the absorbances of blank before and after the heating, respectively. Each activity vs. concentration curve was analyzed statistically according to Tallarida and Murray (Tallarida and Murray, 1986), to determine the concentrations corresponding to a stabilizing activity of 16% (EC_{16}) and 50% (EC_{50}); (ii) HPLC: briefly, each substance was dissolved at a concentration double as that indicated in the tables as the highest concentration tested (S_{MAX}), in 0.02 M Tris (pH 8 at 22°C), serially diluted with the same buffer, and each solution was mixed 1:1 with 0.4% (w/v) HSA and heated for 5 min at 80°C as described above; the pro-tective activity of a substance X at the concentration c was calculated with the formula:

$$a_{X,c} = [(AUC_{X,\ 80°C} - AUC_{0,\ 80°C})/(AUC_{0,\ 22°C} - AUC_{0,\ 80°C})] \cdot 100$$

and each activity vs. concentration curve was analyzed statistically as described above for the turbidimetric assay.

Statistical analysis

Linear regression analyses and Students t tests were performed with the software Sigma-Plot 6.0 (SPSS, Chicago, IL, USA).

RESULTS

Purification of albumin from normal human serum

More than 300 mg of human serum albumin (HSA_{NHS}) were purified from 10 ml of normal human serum (NHS) by affinity chromatography: the purity of this preparation was higher than 96% as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (Fig. 1A) and reversed-phase high performance liquid chromatography (HPLC) (Fig. 1B).

Heat-induced denaturation of albumins

When different concentrations of HSA_{NHS}, HSA_V and HSA_d, at pH close to their isoelectric point (Abramson and Weissmann, 1989; Vane, 1971), were heated for 30 min at 70°C, a strong increase of turbidity was observed

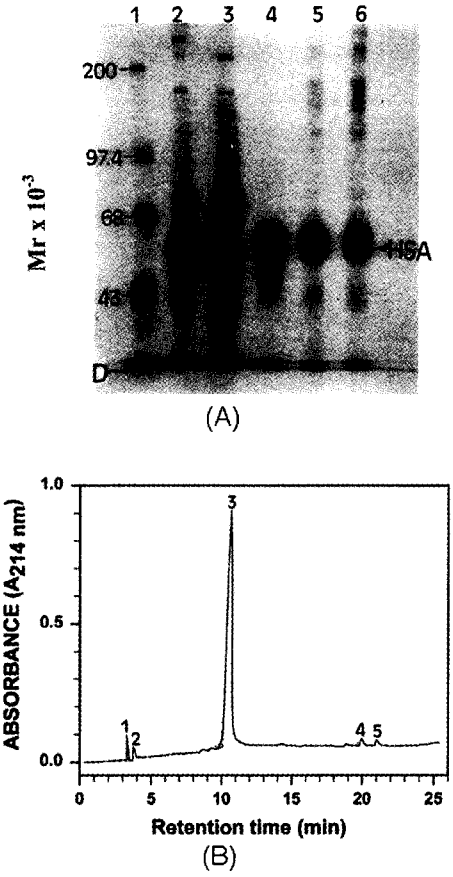

Fig. 1. Purification of albumin from normal human serum. (A) More than 300 mg of human serum albumin (HSA_{NHS}) were purified from 10 ml of normal human serum (NHS) by Affi-gel blue affinity chromatography. Aliquots of about 20 µg of NHS (0.3 µl, lane 2), NHS after albumin removal (lane 3), HSA_{NHS} (lane 4), commercially available fatted (HSA_V: Cohns fraction V, 96% pure, fraction V, lane 5) and defatted HSA [HSA_d: by mild treatment with activated charcoal, according to (47), ≥96% pure, lane 6] were fractionated under reducing conditions (in the presence of 2-mercapto-ethanol) on a 7.5% *T* gel, according to (Laemmli, 1970), and the gel was stained with Coomassie blue R-250. Lane 1 contained the following protein Mr standards: myosin (200 Kd), phospho-rylase b (97.4 Kd), bovine serum albumin (68 Kd) and oval-bumin (43 Kd). (B) About 100 µg of HSA_{NHS} were loaded onto a Vydac C_{18} reversed-phase column equilibrated at 1 ml/min with 20% of solvent A (5% acetonitrile, 95% water and 0.1 % trifluoroacetic acid) in solvent B (95% acetonitrile, 5% water and 0.1% trifluoroacetic acid) and a gradient from 20 to 100% B was started, monitoring the eluate at 214 nm with a sensitivity of 1 A.U.F.S. HSA_{NHS} was eluted under peak 3.

(Fig. 2). It was noted that HSA_{NHS} and HSA_V behaved similarly while the defatted product HSA_d was much more prone to denaturation (Fig. 2).

The higher stability of HSA_V compared to HSA_d was confirmed also by turbidimetry and HPLC: (i) when protein solutions (0.2%) at pH 5.3 were heated for 30

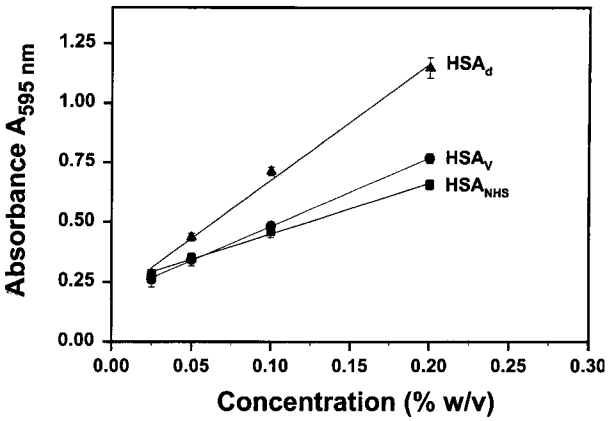

Fig. 2. Heat-induced denaturation of different albumin preparations. Three different human serum albumin (HSA) preparations, purified from normal human serum (NHS) by affinity chromatography (HSA_{NHS}) or obtained from Sigma (HSA_V: Cohns fraction V, 96% pure; and HSA_d: defatted by activated charcoal treatment, ≥96% pure), were dissolved at 0, 0.05, 0.1 and 0.2 % in 0.066 M sodium phosphate (pH 5.3 at 22°C), aliquots of 100 µl were heated for 30 min at 70°C, and the turbidity of the samples was determined at 595 nm, using an automatic microplate reader (model 3550, Bio-Rad, Hercules CA, USA).

min at different temperatures, a significant difference ($p < 0.001$) of turbidity was observed at 65°C, 70°C and 75°C (Fig. 3A); (ii) when protein solutions (0.2%) at pH 8.0 were heated for 5 min at different temperatures, a significant difference ($p<0.001$) of aggregation was observed at 75°C and 80°C (Fig. 3B).

When a 0.2% solution of HSA_V at pH 8 was heated for 5 min at 80°C in the presence of caprylic (10^{-3} M), capric (5×10^{-4} M) and lauric acid (5×10^{-4} M) and protein aggregation was evaluated by PAGE under non-reducing conditions, it was noted that the capric and lauric acid, but not caprylic acid, protected HSA against denaturation and subsequent aggregation (Fig. 4): HSA_V heated in the presence of lauric acid was virtually undistinguishable from the native protein (Fig. 4, lane 3 vs. $C_{22°C}$).

Both FA and NSAIDs, tested at their maximum solubility (S_{MAX}) in the experimental conditions described, were potent inhibitors of heat-induced denaturation of NHS, HSA_V and HSA_d as judged by both the turbidimetric and the chromatographic (HPLC) method (Tables I, II). In particular, we observed that: (i) the stabilization of NHS by FA required higher concentrations compared to HSA_V (Table I, Fig. 6A); (ii) generally speaking, the potency of FA was higher than that of the NSAIDs (Tables I, II, Fig. 5); (c) the stabilization of HSA_d by FA and NSAIDs usually required higher concentrations than that of HSA_V, a phenomenon that was particularly marked for NSAIDs (Tables I, II, Fig. 6B, C); (d) despite the high slope of the regression line (Fig. 6, panel D), the potency of FA in the HPLC assay (Table I) but not that of NSAIDs (Table II, Fig. 6E), was often similar to that observed by

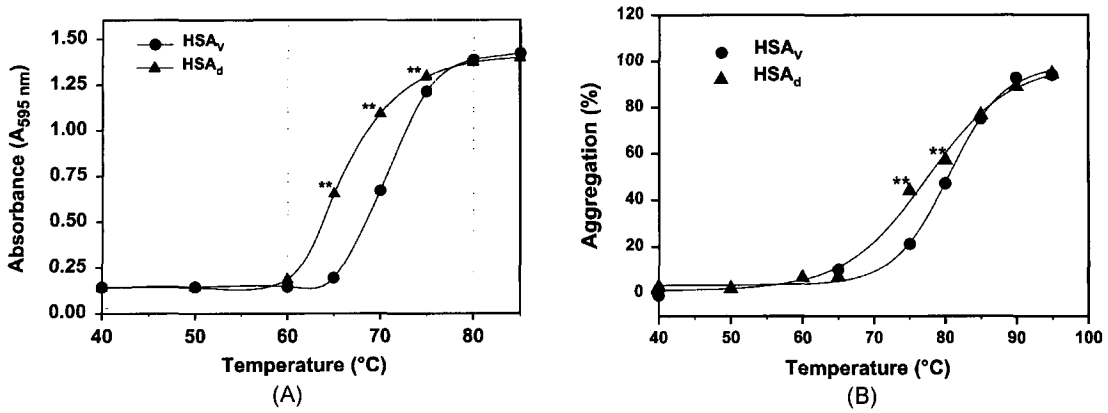

Fig. 3. Heat-induced denaturation of fatted and defatted albumin at different temperatures. (A) Two preparations of fatted (HSA_V: Cohns fraction V, 96% pure) and defatted (HSA_d: defatted by activated charcoal treatment, ≥96% pure) albumins were dissolved at 0.2% (w/v) in 0.066 M sodium phosphate (pH 5.3 at 22°C) and heat-induced denaturation of aliquots of 100 µl was examined at different temperatures in the range 40-95°C. **p<0.001 by the Students t test. (B) HSA_V and HSA_d were dissolved at 0.2% (w/v) in 0.02 M Tris (pH 8.0 at 22°C) and heat-induced aggregation was studied by gel permeation HPLC: aliquots of 0.5 ml were heated in duplicate at different temperatures (40-95°C) for 5 min at 80°C and aliquots of 50 µl (100 µg) were loaded onto a Bio-Sil TSK-250 column equilibrated with TSK-buffer (0.05 M sodium phosphate pH 6.8 at 22 °C, containing 0.15 M NaCl) at the flow rate of 1 ml/min, monitoring the eluate at 280 nm with a sensitivity of 0.1 A.U.F.S. Then, the area under the peak of monomeric albumin (AUC) was measured using an electronic integrator (model 2221, Pharmacia-LKB) and the extent of aggregation was calculated using the following formula: *aggregation* (%)=100·(1-$AUC_{T°C}$/$AUC_{22°C}$). **p< 0.001 by the Students t-test.

turbidimetry (Tables I, II, Fig 6); (e) when the binding constants [K_{a1}, (M^{-1})] of selected FA and NSAIDs [according to (Carter and Ho, 1994)] were plotted against their EC_{16} (HSA_V, turbidimetric), a weak linear correlation was found (r^2=0.16, p>0.05): in fact the stabilizing properties of some NSAIDs, like ibuprofen, were similar to those of

Table I. Inhibition of heat-induced denaturation of normal human serum and albumin by fatty acids

	TURBIDIMETRIC ASSAY							HPLC ASSAY	
	NHSa			HSA_V[b]		HSA_d[c]			
	S_{MAX}[d]	E_{16}[e]	E_{50}[e]	EC_{16}	EC_{50}	EC_{16}	EC_{50}	EC_{16}	EC_{50}
Caprylic acid (C8:0)	1×10^{-3}	≅ S_{MAX}	> S_{MAX}	1.2×10^{-4}	2.8×10^{-4}	2.0×10^{-4}	8.9×10^{-4}	≅ S_{MAX}	> S_{MAX}
Capric acid (C10:0)	5×10^{-4}	-[f]		1.5×10^{-5}	5.9×10^{-5}	4.2×10^{-5}	1.9×10^{-4}	1.8×10^{-4}	≅ S_{MAX}
Lauric acid (C12:0)	5×10^{-4}	1.4×10^{-5}	1.3×10^{-4}	2.0×10^{-5}	6.1×10^{-5}	4.9×10^{-5}	1.5×10^{-4}	2.9×10^{-5}	7.8×10^{-5}
Myristic acid (C14:0)	5×10^{-4}	3.8×10^{-5}	2.1×10^{-4}	1.8×10^{-5}	7.3×10^{-5}	5.6×10^{-5}	1.8×10^{-4}	2.9×10^{-5}	5.3×10^{-5}
Palmitic acid (C16:0)	5×10^{-4}	3.7×10^{-5}	3.0×10^{-4}	2.4×10^{-5}	5.4×10^{-5}	9.9×10^{-5}	≅ S_{MAX}	2.5×10^{-5}	8.2×10^{-5}
Stearic acid (C18:0)	5×10^{-4}	6.4×10^{-5}	≅ S_{MAX}	1.9×10^{-5}	8.2×10^{-5}	2.6×10^{-4}	≅ S_{MAX}	1.4×10^{-5}	7.5×10^{-5}
Oleic acid (C18:1)	5×10^{-4}	3.5×10^{-5}	1.5×10^{-4}	2.6×10^{-5}	8.8×10^{-5}	5.3×10^{-5}	≅ S_{MAX}	1.9×10^{-5}	6.1×10^{-5}
Linoleic acid (C18:2)	5×10^{-4}	5.7×10^{-5}	3.0×10^{-4}	2.3×10^{-5}	1.1×10^{-4}	8.2×10^{-5}	4.6×10^{-4}	-	
γ-Linolenic acid (C18:3, n-6)	5×10^{-4}	1.1×10^{-4}	≅ S_{MAX}	7.1×10^{-4}	2.3×10^{-4}	1.9×10^{-4}	≅ S_{MAX}	-	
α-Linolenic acid (C18:3, n-3)	5×10^{-4}	1.7×10^{-5}	7.5×10^{-5}	3.8×10^{-5}	7.4×10^{-5}	4.1×10^{-5}	1.6×10^{-4}	-	
Arachidic acid (C20:0)	5×10^{-5}	≅ S_{MAX}	> S_{MAX}	≅ S_{MAX}	> S_{MAX}	≅ S_{MAX}	> S_{MAX}	-	
Arachidonic acid (C20:4, n-6)	2×10^{-4}	9.7×10^{-5}	≅ S_{MAX}	1.4×10^{-5}	6.5×10^{-5}	6.6×10^{-5}	≅ S_{MAX}	1.9×10^{-5}	7.0×10^{-5}
Eicosapentaenoic acid (C20:5, n-3)	1×10^{-4}	1.1×10^{-5}	7.8×10^{-5}	1.6×10^{-5}	5.0×10^{-5}	4.3×10^{-5}	≅ S_{MAX}	-	
Docosahexaenoic acid (C22:6, n-3)	1×10^{-4}	1.6×10^{-5}	≅ S_{MAX}	3.3×10^{-5}	≅ S_{MAX}	6.5×10^{-5}	> S_{MAX}	-	

[a]Tested on normal human serum
[b]Tested on fatted HSA (Sigma product N° A-1653)
[c]Tested on defatted HSA (Sigma product N° A-1887)
[d]Highest concentration (M) of fatty acid tested
[e]Effective doses (M), calculated according to Tallarida and Murray (1986)
[f]Not tested

Table II. Inhibition of heat-induced denaturation of albumin by nonsteroidal antiinflammatory drugs

	TURBIDIMETRIC ASSAY				HPLC ASSAY	
	HSA_V[a]		DEFATTECHSA$_d$[b]			
	S^c_{MAX}	EC_{16}	EC_{50}	EC_{16}	EC_{50}	EC_{16}	EC_{50}
A. Salicylates							
Diflunisal	1×10^{-3}	3.3×10^{-5}	8.5×10^{-5}	2.6×10^{-5}	8.2×10^{-5}	-	
Salicylic acid	1×10^{-3}	6.8×10^{-4}	$\cong S_{MAX}$	non-active		non-active	
B. Para-aminophenol derivatives							
Acetaminophen	5×10^{-4}	non-active		non-active		non-active	
C. Indole and indene acetic acids							
Indomethacin	1×10^{-3}	1.5×10^{-4}	3.1×10^{-4}	4.0×10^{-4}	7.9×10^{-4}	1.8×10^{-4}	$\cong S_{MAX}$
Sulindac	2.5×10^{-4}	$\cong S_{MAX}$	$> S_{MAX}$	$\cong S_{MAX}$	$> S_{MAX}$	-	
D. Heteroaryl acetic acids							
Diclofenac	5×10^{-4}	6.0×10^{-5}	1.1×10^{-4}	1.4×10^{-4}	2.7×10^{-4}	2.6×10^{-4}	$> S_{MAX}$
Tolmetin	1×10^{-3}	1.4×10^{-4}	3.8×10^{-4}	1.4×10^{-4}	4.3×10^{-4}	-	
E. Arylpropionic acids							
Fenoprofen	1×10^{-3}	2.5×10^{-5}	5.6×10^{-5}	3.7×10^{-5}	9.9×10^{-5}	-	
Flurobiprofen	5×10^{-4}	1.8×10^{-5}	7.0×10^{-5}	2.7×10^{-5}	1.1×10^{-4}	$\cong S_{MAX}$	$> S_{MAX}$
Ibuprofen	1×10^{-3}	1.8×10^{-5}	1.1×10^{-4}	1.4×10^{-4}	2.8×10^{-4}	1.4×10^{-4}	5.7×10^{-4}
Ketoprofen	1×10^{-3}	1.0×10^{-4}	2.3×10^{-4}	1.9×10^{-4}	5.4×10^{-4}	$\cong S_{MAX}$	$> S_{MAX}$
Naproxen	5×10^{-4}	2.2×10^{-5}	7.6×10^{-5}	1.3×10^{-4}	3.4×10^{-4}	$\cong S_{MAX}$	$> S_{MAX}$
F. Fenamates							
Meclofenamic acid	2.5×10^{-4}	2.3×10^{-5}	6.1×10^{-5}	6.9×10^{-5}	3.1×10^{-4}	-	
Mefenamic acid	1×10^{-4}	1.9×10^{-5}	3.6×10^{-5}	7.6×10^{-5}	$\cong S_{MAX}$	-	
G. Enolic acids							
Phenylbutazone	5×10^{-4}	9.9×10^{-5}	2.1×10^{-4}	$\cong S_{MAX}$	$> S_{MAX}$	$\cong S_{MAX}$	$> S_{MAX}$
Piroxicam	2.5×10^{-4}	5.3×10^{-5}	$\cong S_{MAX}$	$\cong S_{MAX}$	$> S_{MAX}$	-	
H. Alkanones							
Nabumetone	5×10^{-4}	non-active		non-active		non-active	
I. Antidenaturant drugs							
Bendazac	1×10^{-3}	1.4×10^{-4}	4.4×10^{-4}	4.0×10^{-4}	$\cong S_{MAX}$	$\cong S_{MAX}$	$> S_{MAX}$
Bindarit	5×10^{-4}	5.0×10^{-5}	1.3×10^{-4}	1.6×10^{-4}	$\cong S_{MAX}$	$\cong S_{MAX}$	$> S_{MAX}$

[a]Sigma product N° A-1653
[b]Sigma product N° A-1887
[c]Highest concentration tested

many FA, despite their higher affinity for the protein.

DISCUSSION

A survey of the antidenaturant activity of selected fatty acids (FA) and nonsteroidal antiinflammatory drugs (NSAID) was reported: we confirmed and expanded previous studies (Ballou *et al.*, 1944; Boyer *et al.*, 1946; Mizushima, 1964; Mizushima and Suzuk, 1965; Peters, 1985; Saso *et al.*, 1999; Saso *et al.*, 1998) indicating that both classes of compounds are potent inhibitors of heat-induced denaturation of human serum albumin (HSA). In addition, we tested these compounds on normal human serum (NHS) and defatted HSA (HSA_d) using a modified version (Saso *et al.*, 1998; Saso *et al.*, 1999) of the Mizushimas test (Mizushima, 1964) and a new high performance liquid chromatographic (HPLC) method (Saso *et al.*, 1998).

We easily prepared over 300 mg of highly purified HSA (Fig. 1) by a one-step chromatographic procedure but we

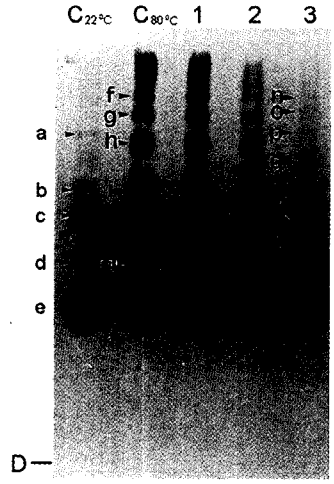

Fig. 4. Effect of selected fatty acids on heat-induced denaturation of albumin evaluated by polyacrylamide gel electrophoresis. HSA_V (Cohns fraction V, 96% pure) was dissolved at 0.2% (w/v) in 0.02 M Tris (pH 8.0 at 22°C) and aliquots of 0.5 ml were heated for 5 min in a water bath at 80°C in the absence ($C_{80°C}$) or presence of caprylic (10^{-3} M, lane 1), capric (5×10^{-4} M, lane 2) and lauric acid (5×10^{-4} M, lane 3). Then 20 µl (40 ug) of each sample were analyzed by PAGE on a 7.5% *T* under non-reducing conditions. $C_{22°C}$: negative control (not heated).

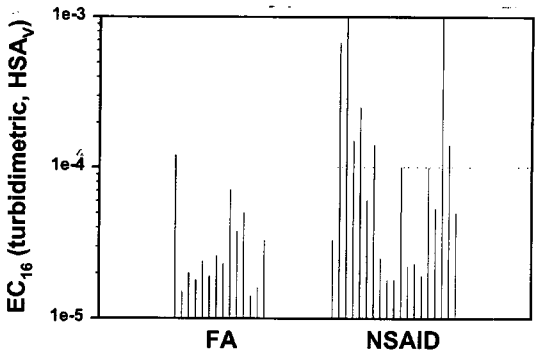

Fig. 5. Effect of fatty acids and nonsteroidal antiinflammatory drugs on heat induced denaturation of albumin. The EC_{16} of selected fatty acids (FA) and nonsteroidal antiinflammatory drugs (NSAIDs) reported in Tables I and II for the turbidimetric assay performed on the fatted commercial albumin (HSA_V: Cohns fraction V, 96% pure), were plotted in the same order as listed.

found that, for practical purposes, it was not very different from the one commercially available (HSA_V): thus, we decided to use the latter for this study, with the advantage of producing data more easily reproducible in other laboratories, reserving the described purification procedure for future studies, such as the evaluation of protein stability in individual patients with rheumatic diseases.

We confirmed previously reported data (Peters, 1985; Saso *et al.*, 1999), indicating the lower stability of defatted albumin compared to the FA-carrying protein (Fig. 3, 4).

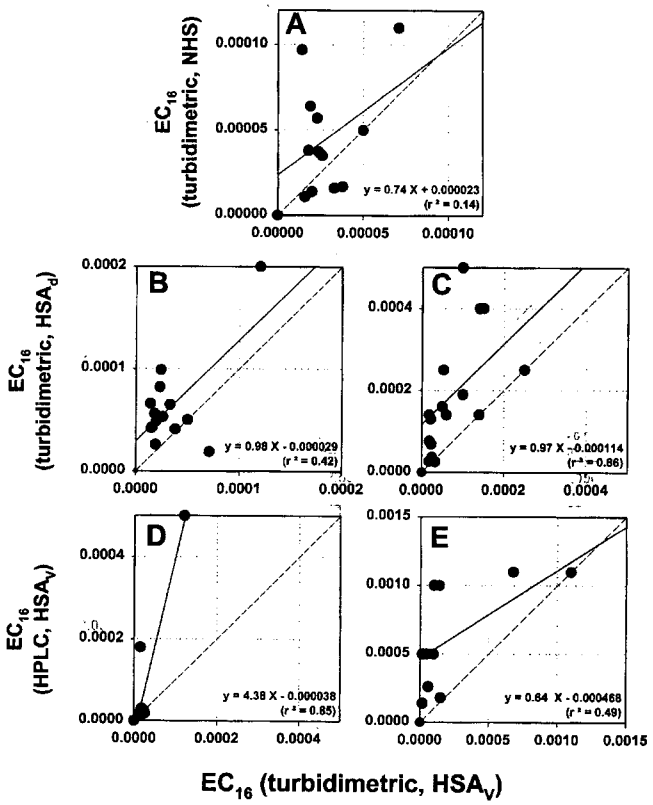

Fig. 6. Effect of fatty acids and nonsteroidal antiinflammatory drugs on heat induced denaturation of normal human serum and albumins. The EC_{16} of selected fatty acids (FA) and nonsteroidal antiinflammatory drugs (NSAIDs) reported in Tables I and II for normal human serum (NHS), fatted commercial albumin (HSA_V: Cohns fraction V, 96% pure), and defatted commercial albumin (HSA_d: defatted by activated charcoal treatment, ≥96% pure) were plotted against each other. — linear regression lines; ---- y=x, reference line.

Actually, we initially hoped that the use of HSA_d could increase the sensitivity of the denaturation assay, soon realizing that the opposite was true: the protein was less stable and a higher concentration of stabilizers was necessary (Tables I, II, Fig. 6B,C); the phenomenon was more evident with NSAIDs compared to FA, probably because these drugs stabilize albumin mainly by binding to two specific sites which are distinct from those of FA (Peters, 1985; Carter and Ho, 1994) (Tables I, II, Fig. 6B, C). Moreover, the potency of FA was usually higher than that of the NSAIDs (Tables I, II, Fig. 5), but the antidenaturant activity of some drugs like ibuprofen, was comparable to that of many FA despite their lower affinity for the protein (Fig. 7), indicating that albumin stabilization by NSAIDs did not depend only on protein binding.

The lower activity of FA toward NHS by FA than HSA_V (Table I, Fig. 6A), could be due the poorer stabilization of serum globulins which bind FA with much lower affinity than HSA, their physiological carrier in the circulation (Peters, 1985).

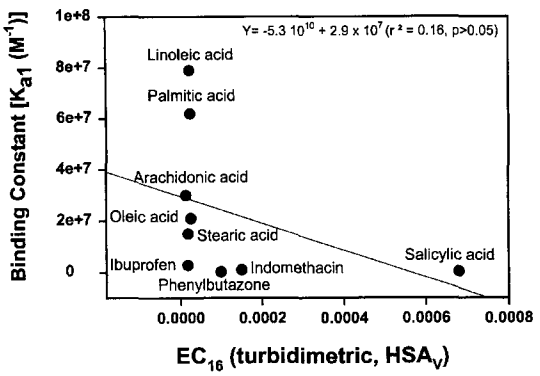

Fig. 7. Correlation between the binding affinity and the anti-denaturant activity. The binding constants [K_{a1}, (M^{-1})] of selected fatty acids (FA) and nonsteroidal antiinflammatory drugs (NSAIDs), reported in (27), were plotted against the EC_{16} listed in Tables I and II for the turbidimetric assay performed on the commercially available fatted albumin (HSA_V). The linear correlation was not statistically significant (r^2=0.16, p>0.05).

Despite the high slope of the regression line (Fig. 6, panel D), the potency of FA in the HPLC assay (Table I) but not that of NSAIDs (Table II, Fig. 6E), was often similar to that observed by turbidimetry (Tables I, II, Fig 6); however, the less sensitive chromatographic method was useful because it confirmed that all substances examined were true inhibitors of albumin aggregation (Saso *et al.*, 1999).

In conclusion, we consider the study of the stabilizing properties of NSAIDs and their analogues relevant from a pharmacological point of view because: (i) protection against albumin denaturation has been used for many years for the screening of new antiinflammatory drugs (Mizushima, 1964; Mizushima and Suzuki, 1965; Nargund *et al.*, 1994); (ii) aspirin and other NSAIDs are known to protect against cataract (Harding, 1992), a condition in which protein denaturation plays a major role, and bendazac is one of the few drugs rationally developed for the pharmacological treatment of this disease (Silvestrini, 1987); (iii) it is generally thought that the efficacy of indomethacin (Rogers *et al.*, 1993) and other NSAIDs in Alzheimers disease is due to the inhibition of cyclo-oxygenase: however it was recently reported that the neuroprotective activity of aspirin was significant only at doses higher than those necessary for this effect (Gomes, 1998); (iv) protein denaturation could be the initial step for other protein modifications such as glycosylation; specific abnormal glycosylation phenomena do occur in chronic inflammatory disorders (Silvestrini *et al.*, 1989; Saso *et al.*, 1993a; Saso *et al.*, 1993b) and were prevented by bindarit in the case of hemopexin (Saso *et al.*, 1992); protein glycation phenomena that occur in cataract (Stevens, 1998) and Alzheimers disease (Colaco *et al.*, 1996) could be inhibited by NSAIDs (Swamy and Abraham, 1989; Roberts and Harding, 1990; Cherian and Abraham, 1993) and bendazac (Bruno *et al.*, 1988; Marques *et al.*, 1995; Chen, 1967).

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