nisolone was not detectable in PFC after 0.5 or 5 hr of agitation. Thus, PFC emulsion bound prednisolone is associated only with the emulsifiers of the emulsion droplets.

In summary, prednisolone was bound by the PFC emulsion through an interaction with emulsifiers. While this binding was relatively weak, prednisolone was significantly bound by the PFC emulsion even in the presence of HSA. This binding partially offset the increase in free prednisolone that occurred upon HSA dilution. Changes in prednisolone binding by HSA and the PFC emulsion upon mixing of their solutions appeared to be due to dilution alone.

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Complement-dependent stimulation of prostacyclin biosynthesis: inhibition by rosmarinic acid

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The complement system forms a defence mechanism concerned with recognition and elimination of microbes [1]. It consists of two cascades of sequentially activated serum proteins which converge at complement factor C3, from which activation may proceed through C5 to the lytic pathway. Activated complement components (C3a, C3b, C5a) mediate inflammatory processes, e.g. by causing chemotaxis, neutrophil activation, histamine release, increased vascular permeability, and by coating of the microbial cell wall with ligands for receptors on phagocytes [1]. In addition, we have shown that C5a and its metabolite C5a des Arg are potent stimuli of prostacyclin biosynthesis in rabbit mesothelial and endothelial cells [2, 3], and suggested that this could represent a mechanism for increasing local blood flow, thus promoting the development of an acute inflammatory response [2, 3].

Pharmacological modulation of the complement system could be of potential interest for the treatment or control of pathophysiological situations linked with complement activation [4]. Recently, it has been discovered that rosmarinic acid (2-[[3-(3,4-dihydroxyphenyl-1-oxo-2-propinyl]-oxy]-3-(3,4-dihydroxyphenyl)-propionic acid, Nattermann), a product extracted and purified from *Melissa officinalis*, inhibits the C3 convertase (C4b2a) of the classical pathway [5]. It is one of the few [4] compounds with *in vivo* activity, since rosmarinic acid (20 mg/kg i.v.) suppressed endotoxin-induced complement activation in a rabbit model of circulatory shock [6]. This was associated with less severe hypotension and thrombocytopenia and diminished generation of prostacyclin and thromboxane A_2 [6]. However, this experiment did not exclude the possibility that rosmarinic acid inhibited cyclo-oxygenase, prostacyclin or thromboxane synthase activities directly.

The purpose of the present experiments was to test whether rosmarinic acid suppressed activation of human serum complement by cobra venom factor (CVF), and whether this would be reflected in diminished stimulation of prostacyclin biosynthesis. CVF selectively activates the alternate pathway (consisting of factors B, D, C3b) by combining with Bb to form a stable C3 convertase (CVF, Bb) which can activate both C3 and C5 [1], in contrast to endotoxin which may activate both pathways as well as other defence systems. Rabbit peritoneal tissue, a rich source of prostacyclin [7] was used for incubation with the activated sera.

Serum from seven volunteers was pooled and made 1 M with ε-aminocaproic acid (fresh serum). An aliquot of this was heated at 56° for 15 min in order to inactivate complement factors C2 and B (heat-inactivated serum). Fresh and heat-inactivated serum were incubated with cobra venom factor (CVF, Sigma, 10 units/ml) for 120 min at 37° in the presence or absence of 1 mM rosmarinic acid. This concentration is based on the estimated initial plasma concentration achieved following i.v. administration of the non-toxic dose of 40 mg/kg, as extrapolated from pharmacokinetic studies in rats. Rosmarinic acid was used in purified crystalline form and dissolved in 0.9% NaCl by addition of 0.1 N NaOH to pH 7. Thereafter, the comp-

lement hemolytic activity (CH50 units) in the activated sera was measured after serial dilution [8]. The CH50 is the dilution of the serum which contains the quantity of complement required for 50% lysis of sheep red blood cells coated with anti-sheep red blood cell antiserum in a hemolytic assay system. In addition, complement factors C3, C4 and C5 were assessed by radial immunodiffusion [9]. The amounts of C5 were compared with a reference serum.

These serum preparations were stored at −70° until they were used for incubation of rabbit peritoneal tissue as described previously [7]. After killing the rabbits, the thin serous membranes of the peritoneal cavity were removed and 30 mg (wet weight) tissue was placed in 1 ml ice-cold serum. A sample (0.1 ml) was taken from the serum, added to 0.9 ml 50 mM Tris–HCl buffer pH 7.5 containing 0.01 mg indomethacin and used for assessment of its prostacyclin content at zero time. Subsequently, the tissue was incubated for 30 min at 37° in a shaking waterbath, and then a second aliquot (0.1 ml) was taken, added to 0.9 ml Tris–HCl buffer with 0.01 mg indomethacin and its prostacyclin content measured. The difference between the two measurements gives the net amount of prostacyclin formed during the 30 min incubation [7]. Prostacyclin was measured by radioimmunoassay of its non-enzymic degradation product 6-oxo-prostaglandin-$F_{1\alpha}$ as described [2, 3, 7].

The hemolytic activity of fresh serum (480 CH50 units/ml), an overall estimate of the functional activity of the classical (C1, C4, C2 and C3) and membrane attack pathway (C5 to C9) was completely destroyed by the heat treatment (<10 CH50 units/ml), whereas the concentration of immunoreactive C3 (iC3) in fresh serum (1400 mg/l) was only slightly diminished by the heat treatment (1270 mg/l). In Fig. 1 the effects of CVF, rosmarinic

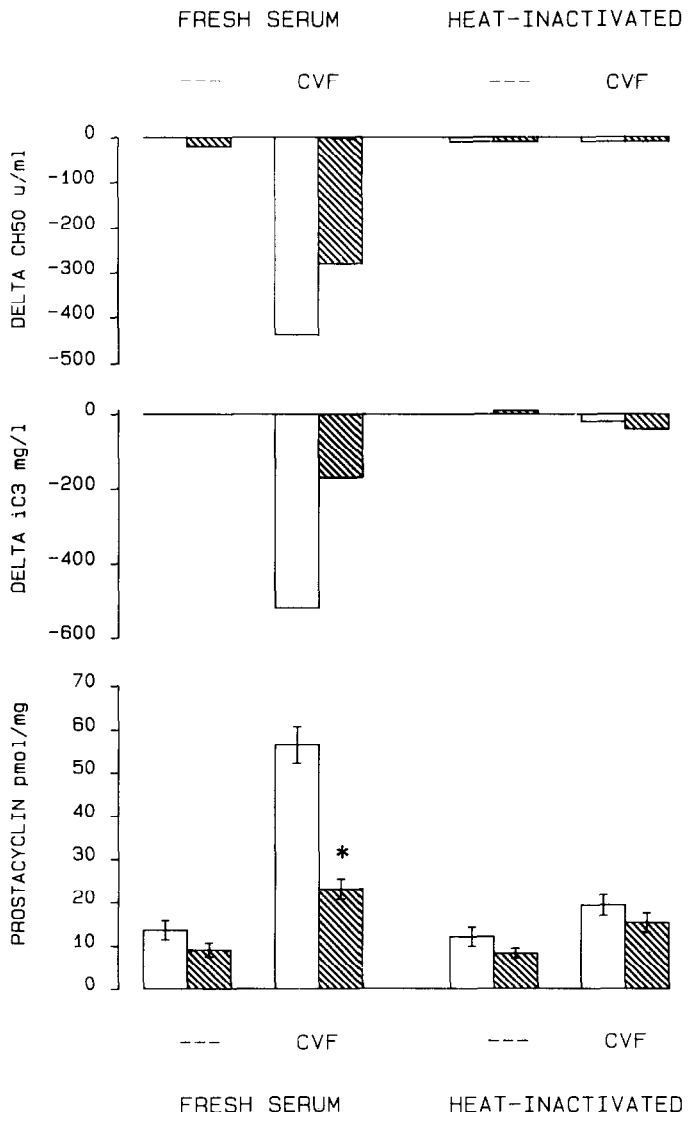

Fig. 1. The influence of 1 mM rosmarinic acid (hatched columns) on the decrease in hemolytic activity (delta CH50) and immunoreactive complement factor C3 (delta iC3) in fresh and heat-inactivated pooled human serum incubated with or without (---) cobra venom factor (CVF, 10 units/ml). The lower part shows the stimulation of prostacyclin biosynthesis by rabbit peritoneal tissue placed in CVF-treated fresh serum, which is counteracted by the presence of rosmarinic acid during the CVF treatment (mean ± S.E.M., N = 12). *$P < 0.05$, rosmarinic acid treated vs the corresponding control, one way analysis of variance ($F_{7,88} = 20.89$) followed by Duncan's New Multiple Range statistic.

acid and their combination on these parameters are summarized. Upon incubation with fresh serum, rosmarinic acid did not affect the concentration of iC3, but caused a small decrease in the hemolytic activity. This cannot be explained by interference of the residual 3–5 μM rosmarinic acid present in the hemolytic assay, since 20 μM rosmarinic acid did not affect this functional test (results not shown). CVF clearly activated complement, as indicated by the almost complete disappearance of hemolytic activity (Fig. 1). In keeping with the activation of the alternate pathway, the levels of iC3 (Fig. 1) and iC5 (from 102 to 90% of the reference serum) were decreased in pooled CVF-treated fresh serum, whereas the concentration of immunoreactive C4 (290 mg/l), a constituent of the classical pathway, remained unchanged. The activation of the alternate pathway was suppressed by 1 mM rosmarinic acid, which was apparent from the diminished consumption of CH50, iC3 (Fig. 1) and iC5 (from 102 to 95% of the reference serum). The incubation of CVF and/or rosmarinic acid with heat-inactivated serum did not change any of the complement parameters (cf. Fig. 1, other results not shown).

These eight (differently treated) sera were used as media for the incubation of rabbit peritoneal tissue (Fig. 1). The net amounts of prostacyclin produced during 30 min incubation with fresh or heat-inactivated serum were not different. The biosynthesis of prostacyclin was clearly stimulated in CVF-treated serum, confirming our results with rabbit serum complement, activated with zymosan, CVF or endotoxin [2]. The stimulation was largely absent in heat-treated serum (Fig. 1), in which factor B had been destroyed [1]. This indicated that the purified CVF preparation itself did not significantly stimulate prostacyclin biosynthesis, which ruled out possible contaminating traces of phospholipase A_2 activity (from the crude cobra venom) as an explanation for the stimulation seen with activated fresh serum. The presence of rosmarinic acid during the activation with CVF strongly reduced its prostacyclin stimulating activity. This is in accordance with the diminished activation of complement factors C3 (Fig. 1) and C5 in the presence of rosmarinic acid, since the prostacyclin stimulating principles are derived from C3 and/or C5 [2, 3, 10]. Rosmarinic acid itself showed a tendency to reduce peritoneal prostacyclin biosynthesis in sera in which the complement system had not been activated (fresh serum, heat-treated serum, or heat-treated serum with CVF), but these effects were statistically not significant. Therefore, it is unlikely that the diminished peritoneal prostacyclin formation in serum activated in the presence of rosmarinic acid can be attributed to direct inhibition of cyclo-oxygenase or prostacyclin synthase activities in the mesothelial cells.

Radiochemical measurements of the conversion of 3 and 30 μM arachidonic acid to the cyclo-oxygenase products thromboxane B_2 and 17-hydroxyheptadecatrienoic acid (HHT) in four preparations of washed rabbit platelets [11, 12] indeed confirmed that 1 μM to 1 mM rosmarinic acid did not interfere with cyclo-oxygenase activity, in contrast to 0.1–1 μM indomethacin (results not shown). The effects of rosmarinic acid on prostacyclin synthase were also tested using the radiochemical conversion of 17 μM arachidonic acid by ram seminal vesicle microsomes [13]. Lower concentrations of rosmarinic acid (1 and 10 μM) stimulated the biosynthesis of prostacyclin (by about 50%, results not shown), possibly by protecting prostacyclin synthase as described previously for the anti-oxidants propylgallate and ascorbic acid [13]. In analogy with these antioxidants, higher concentrations of rosmarinic acid (0.1 and 1 mM) enhanced the non-enzymatic formation of $PGF_{2\alpha}$, probably by a direct reducing effect on PGH_2 and a decreased conversion of arachidonate was observed at 1 mM rosmarinic acid. Both changes were at the expense of the formation of hydroxy fatty acids, PGE_2, PGD_2 and prostacyclin from PGH_2 (results not shown), but the data did not indicate that rosmarinic acid inhibited prostacyclin synthase. An antioxidant action of rosmarinic acid has recently been confirmed by its ability to inhibit chemiluminescence and H_2O_2 generation from human granulocytes *in vitro* [14].

In view of the diminished CVF-induced consumption of C3, it is concluded that rosmarinic acid interfered with the C3 convertase activity of the alternate pathway, which is in accordance with the reported inhibition of the classical C3 convertase [5]. Furthermore our results support the hypothesis that complement-derived principles provide a trigger for the biosynthesis of oxygenated arachidonic acid metabolites in response to an inflammatory stimulus. It appeared that rosmarinic acid can indeed suppress prostaglandin formation via a mode of action different from the non-steroidal anti-inflammatory drugs (NSAIDs), as it did not interfere with cyclo-oxygenase activity. In this respect modulators of complement activation such as rosmarinic acid may have the advantage over NSAIDs as well as glucocorticoids that the inhibition of prostanoid release is specifically restricted to inflammatory sites where complement activation is taking place, unlike NSAIDs or glucocorticoids which may cause side effects by inhibiting eicosanoid formation throughout the organism (e.g. in stomach, kidney, platelets etc.). In analogy to corticoids, inhibitors of complement activation will exert several anti-inflammatory effects in addition to reducing eicosanoid release. Complete inhibition of complement may have the potential risk of depriving the organism of an important defence system. However, modulation of this system could be of therapeutic interest to control excessive or uncontrolled complement activation, e.g. in shock [cf. 6] or diseases with involvement of complement in tissue destruction. Such a temporary modulation could certainly be beneficial during cardiopulmonary bypass or hemoperfusion, during which complement is activated on the dialysis membranes and the C5a returning to the venous circulation is believed to be the trigger of the subsequent pulmonary distress [15].

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Altered responsiveness to alpha- and beta-adrenoceptor stimulation in hepatocytes cultured in defined medium

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Catecholamines regulate important metabolic pathways such as glycogenolysis and gluconeogenesis through alpha and beta adrenoceptors in the livers of a variety of species. In the adult rat liver, it is now well documented that the activation of glycogen phosphorylase by catecholamines is predominantly a cAMP-independent process mediated by alpha$_1$-adrenoceptor stimulation and is thought to be the result of an elevation in the concentration of intracellular Ca^{2+} [1–10]. However, beta-adrenoceptor-mediated activation of glycogen phosphorylase, mediated by cAMP-dependent processes, plays a relatively minor role in the adrenergic activation of glycogenolysis in normal adult rat liver. This pattern of adrenergic activation in adult rat liver with alpha much more prominent than beta is not fixed and static. Indeed, this pattern has been shown to change in a number of physiological and pathological conditions, including adrenalectomy [11], hypothyroidism [12], regeneration [13], cholestasis [14] and preneoplasia [15]. Also, it has been found that beta adrenergic activation of glycogen phosphorylase is more marked in livers from young rats [16].

Recently, it was reported that the relative activities of alpha and beta agonists are progressively reversed during the *in vitro* culturing of isolated hepatocytes [17, 18]. The primary role of alpha adrenoceptors in activating glycogen phosphorylase decreases and an effective beta response, absent in freshly isolated cells, emerges after primary culture. Our studies were designed to investigate the possibility that changes in alpha and beta receptors might underline these alterations in responsiveness.

Materials and methods

Materials. Unlabeled (±)-cyanopindolol was a gift from Dr. G. Engel of Sandoz (Basel, Switzerland). [^{125}I]Cyanopindolol ([^{125}I]CYP) was prepared essentially as described by Engel *et al.* [19]. Carrier-free Na^{125}I (Catalog No. IMS 30) was purchased from Amersham (Arlington Heights, IL). Alpha-D-[U-^{14}C]Glucose-1-phosphate and [^{3}H]prazosin were purchased from New England Nuclear (Boston, MA). Anti-cAMP* rabbit antiserum were obtained from Becton Dickinson. Prazosin was a gift from the Pfizer Pharmaceutical Co. Collagenase (Type II) was from the Worthington Biochemical Co. (Freehold, NJ); newborn calf serum, tissue culture medium 199 (Earl's salt), and DMEM were from Gibco. Other chemicals and reagents were purchased from standard commercial sources.

Isolation of hepatocytes. Male Sprague–Dawley rats, weighing 240–260 g, that had been fed *ad lib.* were used. Hepatocytes were isolated by the collagenase perfusion method of Berry and Friend [20] as modified by Bissell and Guzelian [21]. The yield of cells/liver averaged 1.5×10^8, with 90–95% viability as estimated by trypan blue exclusion.

Fresh hepatocytes. Freshly isolated hepatocytes (6 to 15×10^6 cells) were incubated in plastic tubes in a total volume of 1 ml of Hepes-buffered medium (134 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM Hepes, pH 7.4) supplemented with 40 mM glucose and shaken in an orbital water bath shaker at 150–200 rpm for 30 min at 37°. At the end of the incubation, 200-μl aliquots of the cell suspension were transferred to plastic tubes containing 50 μl of drugs as indicated. The tubes were then shaken in a bath at 37° for 2 min [19] and immediately immersed in liquid nitrogen to terminate the reaction. The frozen hepatocytes were stored at −80° until assay. For the glycogen phosphorylase assay, the frozen hepatocytes were first mixed with a half volume of 30 mM MOPS, 150 mM NaF, 15 mM EDTA and 3 mM dithiothreitol (pH 7.0). The samples were homogenized with a Polytron cell disrupter (Brinkmann Instruments, Westbury, NY) at setting 8 for 20 sec. The homogenates were centrifuged at 12,000 *g* for 5 min at 4°, and the supernatant fraction was used for the assay of glycogen phosphorylase and cAMP as described below.

Cultured hepatocytes. For cultured hepatocytes, Medium 199 was supplemented with the following: 100 units/ml penicillin G; 100 μg/ml streptomycin sulfate: 26 mM $NaHCO_3$; an additional 10 mM glucose (final concentration, 15.6 mM); 3×10^{-8} M crystalline insulin; and 1×10^{-6} M corticosterone. Approximately 4×10^6 cells in a final volume of 3.0 ml were placed in a 60 mm culture dish coated with collagen that had been purified and solubilized by the method of Wood and Keech [22]. The cultures were maintained at 37° in an atmosphere of 95% air and 5% CO_2. The cultured medium was changed to the Hepes-buffered medium described above supplemented with 40 mM glucose at the end of 24 hr. Culture dishes were then incubated at 37° in an atmosphere of 95% air and 5% CO_2 for 30 min. After this preincubation, 50-μl aliquots of various drugs were added to the culture dishes and incubated for 2 min at 37°. At that point, the medium was immediately discarded and the dishes were placed on the dry ice to terminate the reaction. The frozen hepatocytes attached to the dish were stored at −80° until assay.

* Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; [^{125}I]CYP, [^{125}I]cyanopindolol; MEM, Minimum Essential Medium; A23157, calcium ionophore, A23187; IBMX, 3-isobutyl-1-methylxanthine; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and DMEM, Dulbecco's Minimum Essential Medium.