ALTERATION OF ARACHIDONIC ACID METABOLISM WITH SPLEEN MICROSOMES OF IRRADIATED RATS

J. Maclouf*, P. Bernard*, M. Rigaud**, G. Rocquet* and J. C. Breton**

* Division de Radiochimie CRSSA, 1 bis rue du Lt R. Batany 92141, Clamart, France
** Laboratoire de Biochimie CHU Dupuytren, 2 Av. A. Carrel 87031, Limoges Cedex, France

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SUMMARY

Arachidonic acid is metabolized by a rat spleen microsomes cyclooxygenase into prostaglandin D_2, thromboxane B_2, 12-hydroxy-5, 8, 10-heptadecadienoic acid and by a lipoxygenase into 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid and other unidentified compounds as analyzed by a radiometric thin-layer chromatography method and by gas-chromatography-mass spectrometry. This conversion is modified when spleen microsomes are obtained from whole body irradiated rats. Furthermore, if exogenous cofactors are added to the incubation medium, other changes appear that are different for the lipoxygenase and the cyclooxygenase activities. The results suggest a regulatory role of cofactors on both enzymes and/or a modification of sensitivity of the microsomal fraction from irradiated rats to effectors.

INTRODUCTION

It has been shown that the metabolism of arachidonic acid was not only limited to the apparition of PG, but also produced a great number of other derivatives that may play a major active role and represent in some organs a larger proportion of synthesized products than the primary PG (1, 2). Furthermore, in some organs this transformation results from an equilibrium between the lipoxygenase (hydroxylated fatty acids) and the cyclooxygenase (cyclized products) activities (1, 3). It was reported previously that ionizing radiations modified the PGE_2 and $PGF_2\alpha$ content of the mouse spleen (4). One major effect of ionizing radiation is the production of

Abbreviations : PG, prostaglandins
HHT, 12-hydroxy-5, 8, 10-heptadecadienoic acid
HETE, 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid

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radicals amplified by the "oxygen effect". Since the transformation of arachidonic acid is accompanied by peroxidative processes that are membrane bound it seemed interesting to study the conversion of this acid by microsomes obtained from the spleen of irradiated animals. The conversion of arachidonic acid by the rat spleen microsomes of irradiated animals during 9 days after irradiation at lethal dose was studied. The synthesized products were analyzed by gas-chromatography-mass spectrometry. The arachidonic acid conversion was greatly enhanced during the first days after irradiation when compared to spleen microsomes from normal rats ; afterwards it was totally depressed. This study has brought some interesting preliminary results concerning the regulation of arachidonic acid metabolism and may be a novel approach for the study of radiation damage.

MATERIALS AND METHODS

(1-^{14}C)-arachidonic acid (50 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, England. Arachidonic acid (99% purity), L-epinephrine bitartrate and reduced glutathione were purchased from Sigma Chemical Co. Thin layer chromatography plates of silicagel, F 1500, 0.25 mm of thickness were supplied by Sleicher & Schüll. All solvents, from E. Merck were of the best quality available.

Irradiation : 20-30 COBS/CD male rats weighing 120-140 g were exposed to 900 R of ^{60}Co radiations at a dose rate of 41.5R/min, at a target distance of 100 centimeters. This dose corresponded to the LD100 when estimated by survival 30 days after exposure.

Preparation of rat spleen microsomes : After irradiation, 2 rats were killed by decapitation with 2 corresponding normal rats ; this procedure was repeated from day 1 to day 9 after irradiation. The spleens were rapidly collected and placed in pre-weighed flasks containing 5-7 ml of ice cold 0.05 M Tris HCl buffer, pH7.5, weighed, and rapidly homogenized at 0°C using a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 10.000 rpm for 15 min. The cloudy supernatant was then centrifuged at 100.000 g for 1 hour. The microsome pellets were gently suspended by homogenization with a glass teflon homogenizer in cold 0.05 M Tris HCl buffer and were adjusted to a protein concentration of 10 mg/ml as measured by a Lowry method (5) and immediately used for the conversion experiments.

Conversion of arachidonic acid : The simultaneous determination of PG D_2, thromboxane B_2, HHT, HETE and other compounds by thin-layer radiochromatography was based on assay method of Flower et al (6). The following components at the indicated concentrations were incubated 10 min. at 37° in tubes containing final volume of 0.5ml 0.05 M Tris HCl pH 7.5 : arachidonic acid 1.3 nmole (and ^{14}C arachidonic acid, 0.05μCi) and microsome preparation, 3-4 mg protein per tube. The concentration of the cofactors

depended upon the experiment. After incubation, the tubes were immediately chilled and acidified to pH 3 with a few drops of a 0. 625 M citric acid solution and extracted 3 times with 1 ml of Ethyl Acetate. The tubes containing the extracts were then evaporated to dryness in dark under nitrogen. The content of each tube was redissolved in 50μl of ethanol and spotted on thin layer plates. The plates were then developed in the dark with freshly prepared ethyl acetate/water/isooctane/acetic acid (11:10:5:2). Unlabelled standards of PGE_2, $F_2\alpha$, D_2, were run on the same plate. Every week a control of the isotopic dilution of arachidonic acid was submitted to the same conditions.

Quantitative estimation : The thin layer chromatographic plate was analyzed using a Chromelec 101 apparatus (Numelec, Versailles, France). Integration of the total radioactivity distributed on the plate and of individual peaks was done using a Plurimat multi-4 (Intertechnique). The percent of the total radioactivity on plate of each product corresponded to the amount of synthetized compound from the initial substrate content. All results were expressed in picomoles of product per mg of protein for 10 min. incubation.

Identification of the reaction products : In order to confirm or to determine the identity of the biosynthesized products, a large scale reaction was carried out with 50 mg of rat spleen microsomal protein incubated with 60μg of arachidonic acid, 1μCi of (^{14}C)-arachidonic acid and an appropriate amount of cofactors. All Conditions and analysis details used for the identification of each of these compounds will be described elsewhere (9). Gas chromatography-mass spectrometry analysis were carried on a LKB 2091 mass spectrometer equipped with a 25 m glass capillary column coated with polymethyl-siloxanes chemically bonded to glass (7, 8). Theoretical plates per meter : 2817 for C24 at 230°C.

RESULTS AND DISCUSSION

A typical pattern of the thin-layer radiochromatogram of the products of oxygenation of the (^{14}C)-arachidonic acid is shown in figure 1. All the main radioactive products were analyzed by GC-MS analysis. The methyl ester TMS ether derivatives showed a C value of 2755, 36 for compound I, 2751, 22 for compound II, 1924, 43 for compound III, 2128, 4 for compound IV. The mass spectra obtained were respectively m/e 600 (molecular ion) and mainly 529, 510, 439, 420, 301, 295, 256, 225 for compound I which corresponded to the fragmentation obtained with the thromboxane B_2 standard ; for compound II, m/e 539 (molecular ion) and 524, 508, 449, 418, 368, 295, 225 which corresponded to the fragmentation of the PGD_2 standard. Compounds III and IV had respectively m/e 366, 351, 335, 295 and m/e 406, 391, 375, 295 which corresponded to HHT and HETE (1).

The multiple ion analysis of fractions III and IV showed an important discrepancy between the amount of HHT and HETE as calculated using 12-

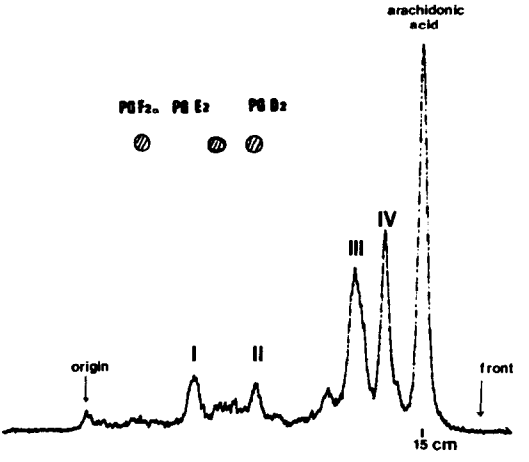

Fig. 1 Thin-layer radiochromatogram of products formed by 10 min. incubation at 37° of rat spleen microsomes (5 mg proteins) with isotopic dilution of (1 - ^{14}C) arachidonic acid, 2.2 nanomoles.

hydroxy-stearic acid as external standard and the isotopic dilution estimation. Since in the presence of indomethacin (a cyclooxygenase inhibitor), there still was radioactivity at peak III, it was concluded that other products synthesized by the lipoxygenase co-migrated with compound III and IV. The identification of these compounds is not complete (9). Our results will then be expressed in terms of I, II, III and IV as a function of the days after irradiation. Figure 2 shows three series of these representations that were made from separate experiments with variable cofactor concentrations in the incubation medium (i. e. epinephrine and reduced glutathione). Almost all synthesized products of spleen microsomes from irradiated animals were affected during day 1 to day 5 after irradiation. Thromboxane B_2 values (I), fluctuated during day 1 to day 5 after irradiation (figure 2a, 2b) or were increased (figure 2c). PGD_2 production (II) seemed little affected. Compounds III (cyclooxygenase - HHT - and lipoxygenase activities) seemed to be synthesized in higher amounts from day 1 to day 5 after irradiation and then getting dramatically depressed. Compound IV (i. e. lipoxygenase activity) is the first compound to be depressed. However, its synthesis by microsomes from irradiated animals is greatly affected by the cofactor concentrations (figure 2a, 2b, and 2c). These results show that the spleen microsomal metabolism of arachidonic acid is strongly altered by ionizing

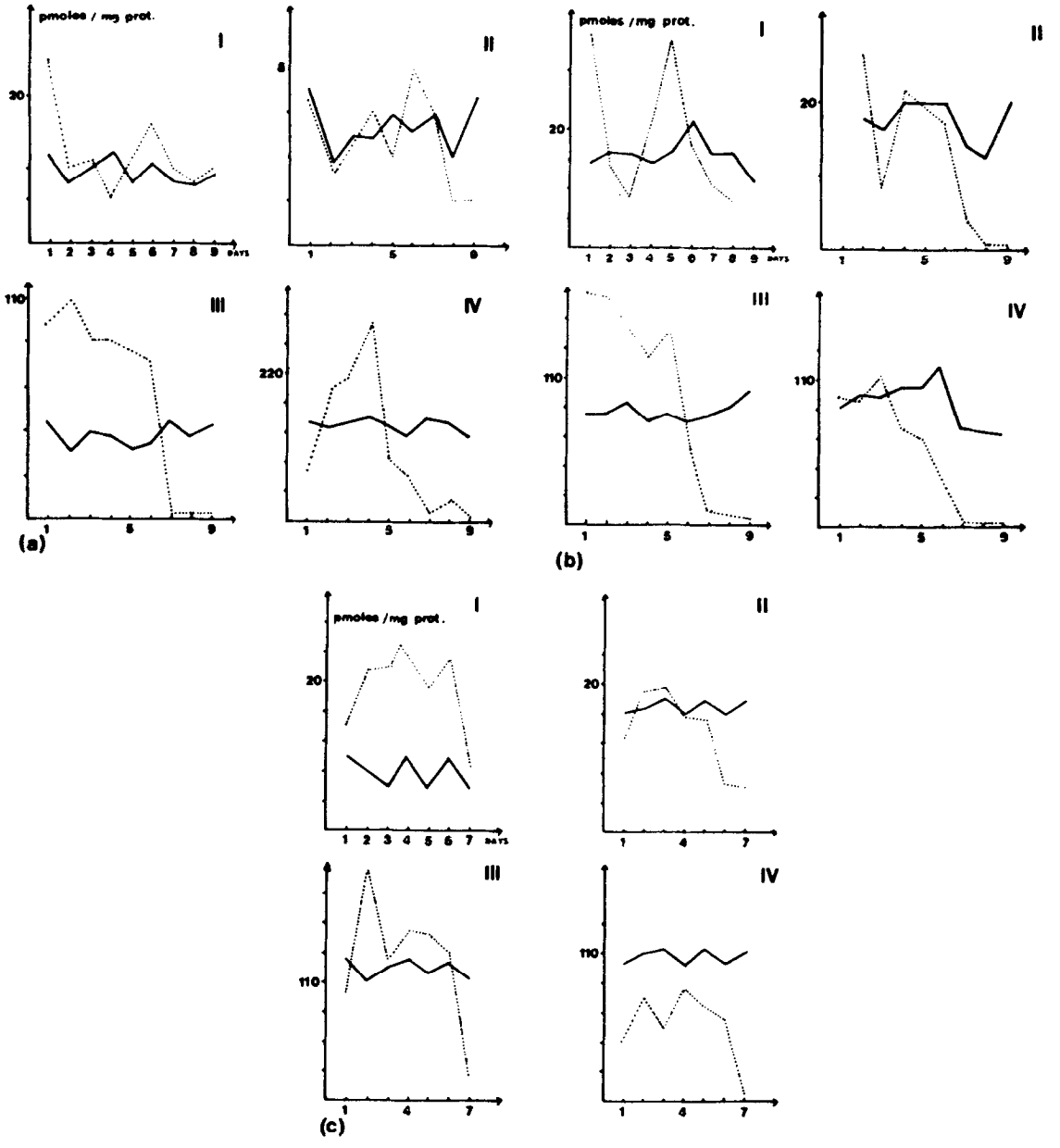

Fig. 2 Metabolism of arachidonic acid by rat spleen microsomes of normal and irradiated animals. The results are expressed in picomoles product/mg protein/10 min. (normal ._. and irradiated. . . . .) as a function of the days following irradiation. Arachidonic acid concentration was 1.3 nanomoles ; protein concentration, 3 mg.

a. L-epinephrine 0.25 μmoles, reduced glutathione 0.125 μmoles
b. L-epinephrine 0.25 μmoles, reduced glutathione 5 nanomoles
c. absence of cofactors.

radiations. Since the oxidation of arachidonic acid is the result of peroxidative enzymes and requires both very reactive oxygen species (10) and some undetermined endogenous reductors that would limit the reaction extent (11), the oxidizing effect of radiation might influence these mechanisms. It can also be thought that natural microsomal quenchers such as -SH groups or other cofactors might be oxidized or modified during exposure to radiations resulting in an increased oxygenation of arachidonic acid. Of particular interest is the fact that these alterations are different considering the concentration of epinephrine and reduced glutathione used throughout the 3 experiments (see figure 2, legend 2 a, b, c). Since the same type of microsomes preparation was used during the different irradiations, this phenomenon is unrelated with the microsomes heterogeneity. The results obtained throughout the different experiments suggest different regulatory mechanisms of the cyclooxygenase and of the lipoxygenase which are highly dependent on cofactor concentrations. It is tempting to think that the concentration of the endogenous pool of reductors (i. e. that control the reaction) was more elevated for normal microsomes than the deprived irradiated microsomes. Therefore , normal microsomes may not be as sensitive to the addition of variable cofactor concentrations. Until now, little is known considering the role played by natural cofactors in the regulation of the arachidonic acid metabolism. In these experiments, we have used the usual effectors that are added to the incubation medium of the microsomal fraction, epinephrine and reduced glutathione. However, when the concentration of these cofactors varied, the metabolism of arachidonic acid by the cyclooxygenase and by the lipoxygenase was differently affected. Furthermore this change of concentration altered unevenly the microsomal metabolism from normal and irradiated rats. The alteration of bioconversion could be explained by a modification of the peroxydative biochemical processes of the first initiating steps of arachidonic metabolism and/or by a modified sensitivity of the enzyme to cofactors after irradiation. The lack of activity which occurs on day 6 may reflect the general decreased protein synthesis of the organ, due to the high radiosensitivity of lymphoid cells or of the platelets.

This first approach of arachidonic acid metabolism in a radiosensitive organ seems promising both in the understanding of the biochemical regulation of

arachidonic acid metabolism by the lipoxygenase and by the cyclooxygenase and for the study of the radiation damage caused by the "oxygen effect". Both of these aspects are being investigated in our laboratory.

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