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4-Hydroxynonenal and Cell Signalling

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The 4-hydroxy-2,3-trans-alkenal series was first discovered by Schauenstein and his group among the lipid peroxidation products in aqueous systems (for a review see[1]).

As it was already known that lipid peroxidation is low in tumors, Schauenstein's idea was to test the toxicity of the aldehydes, and especially of the new series, against tumors. As the synthesis of the natural compounds was at that time impossible, Schauenstein and his group tested the antitumoral activity of 4-hydroxypentenal, a non-natural aldehyde of the same series, that was easier to synthesize. With this aldehyde, the authors were able to show a decrease in cell proliferation and got some results even in the therapeutical attempts.[2]

My collaboration with Trevor Slater had started several years ago when we decided that it was very necessary to investigate deeper the toxicity of the products of lipid peroxidation. At that time (the early 1970s) it was thought that lipid peroxidation produced cell damage by two main mechanisms, i.e. the anatomic impact on cell membranes and the involvement of protein and nucleotide molecules in reactions with lipoperoxides.

When we knew that in Graz, 4-hydroxypentenal had been synthesized, Trevor succeeded in repeating the synthesis in Brunel. So, our first results on aldehydes toxicity were obtained mainly by using this aldehyde.[3] The used substance, however, was not a product of lipid peroxidation itself. Moreover, it was still uncertain that 4-hydroxalkenals were really produced during lipid peroxidation induced in microsomes or in isolated hepatocytes by CCl_4 or by iron.

We decided therefore to try to attract in our collaboration Schauenstein and his group. Schauenstein gladly accepted, and in Venice we had an informal meeting in order to plan the experiments for next 3–4 years. I succeeded in introducing to the group my former collaborator Mario Comporti, at that time professor in Siena. It was in Venice that I met Hermann the first time. Our friendship and strict collaboration continued until his recent death.

The first point of the collaboration was an attempt to identify the toxic compounds present in the dialysate from peroxidizing microsomes. Mario Comporti and his group tried to separate the toxic products by paper chromatography and

realized that most of the toxicity was concentrated in two yellowish spots on the paper. Comporti's scholar Benedetti was sent to Graz, and so 4-hydroxy-nonenal was identified in the major spot, and 4-5-dihydroxydecenal in the smaller one.[4,5]

The collaboration in Vienna among Brunel, Turin and Graz led to the identification of a number (about 25) of carbonyl substances arising from microsomal lipid peroxidation. The samples were collected in Brunel, Graz and Turin, and then exchanged among the different laboratories, and separated by HPLC. It was in Graz, however, that Hermann did the identification of the different compounds by using a mass spectrophotometer combined with a HPLC device.[6,7] At this point, it was clear that lipid peroxidation could produce cell damage even by a third mechanism, i.e. the production of toxic aldehydes. The aldehydes are more diffusable than free radicals, so they were good candidates for exporting damage in organism regions far from the production sites. Aldehydes, however, are quickly removed by cells, especially due to their metabolism, that is afforded by several enzymatic systems, that we studied in several pathological conditions. Moreover, aldehydes quickly react with molecules containing –SH or NH_2 groups. In order to accept that aldehydes could produce damage in remote sites, it was necessary to discover if there was any mechanism to protect them from enzymes and to carry them.

Hermann succeeded in synthesizing 4-hydroxynonenal, and generously dispensed it to us, as well as to other scientists. I was the pathologist of the group, so my job became to study the toxicity of the new substance[8]. We used concentrations of the aldehyde from 1 mM to 1 μM. With concentrations in the range from 10 μM to 1 mM we saw that the aldehyde produced a lot of inhibitions to different cell systems (Table I). As concentrations in the range of 10 μM can be reached in severe CCl_4 poisoning,[7] it became so clear that 4-hydroxynonenal can participate in cell damage. In principle, one has to consider that one molecule of aldehyde is sufficient to bind to an –SH-group, so inactivating it. The demonstration of the several toxic activities of the aldehyde was, therefore, not surprising. The surprise arrived, however, when we used concentrations of aldehyde lower than 10 μM. It had been found in 1979[9] that CCl_4-treatment strongly increases cAMP content of the liver. This might have been due either to an increased synthesis by adenylate cyclase, or to a decreased destruction by the specific phosphodiesterase. As plasmamembranes contain adenylate cyclase, we decided therefore to use this cell organelle as a target for 4-hydroxynonenal action. High concentrations were again inhibitory, but concentrations of 0.1–1 μM were strongly stimulatory. In fact, adenylate cyclase activity increased by about 200% within one minute after addition of the aldehyde, like in the case of stimulation afforded by hormones, as, for instance, glucagon.[10, 11]

The adenylate cyclase system is composed of a catalytic unit, that becomes activated or inhibited by two different types of G-proteins, usually referred to as G_s (stimulatory) and G_i (inhibitory). The G-proteins of both types result from

TABLE I Damaging effects of high concentration of 4-hydroxynonenal on cell enzymes and functions (active concentration are from 10 μM to 1 mM)

Block in protein synthesis
Inhibition of mitochondrial respiration
Inhibition of cytochrome P-450-depending microsomal oxidations
Inhibition of lysosomal enzymes
Inhibition of glucose-6-phosphate dehydrogenase
Inhibition of Golgi galactosyltransferase
Block in secretion of lipoproteins and proteins
Block in tubulin polymerization
Inhibition of ornithine decarboxylase
Loss of cell viability
Increased influx of extracellular Ca^{2+} inside hepatocytes
Inhibition of Ca^{2+}, Mg^{2+} and of Na^+, K^+-adenosine triphoshatases
Inhibition of plasmamembrane 5′-nucleotidase
Inhibition of plasmamembranes adenylate cyclases
Inhibition of phagocytosis

the assembly of three different subunits (α, β and γ). The activation of the system results from the separation of α from $\beta\gamma$. This happens usually under the effect of a signal starting from specific receptors (R_s and R_i respectively) when these bind an agonist. In the case of R_s a well-known agonist is glucagon and in the case of R_i propanol or related subtances are good agonists. G_s is activated also under the influence of cholera toxin, whereas G_i is inhibited in the presence of pertussis toxin. Both toxins, therefore, produce activation of the system. Forskolin also activates adenylate cyclase, but it acts directly on the catalytic unit. By using 50% active concentrations of each of such stimulators together with 50% active concentration of 4-hydroxynonenal, we saw that there is no potentiation, both in the activity and in adenosylribosylation of proteins, between the aldehyde and glucagon, cholera toxin and forskolin, whereas there is a net potentiation in the case of pertussis toxin. It was concluded that stimulation of adenylate cyclase by 4-hydroxynonenal and related aldehydes occurs via the inhibition of G_1. We are now attempting to repeat these experiments on isolated G-proteins.[12]

In my opinion, this is the very first demonstration of a signalling property of a product of lipid peroxidation. It is noteworthy that only the aldehydes of the 4-hydroxy-2,3-trans unsaturated series behave in this way and that the non-hydroxylated or saturated aldehydes do not display such effects. The second surprising result seen with low concentrations of these aldehydes was the demonstration that they display chemoattractant activities towards polymorphonuclear leukocytes. The most active under this point of view is 4-hydroxyoctenal, whose activity can be demonstrated even at the concentration 10^{-11} M, but probably the most important under the quantitative point of view is 4-hydroxynonenal, that is produced in much higher amounts during microsomal lipid peroxidation.

The idea that the products of lipid peroxidation could influence chemotaxis came in my mind when I was listening to a talk by the French pharmacologist Jean Paul Giroud, who had described a new method to measure chemoattraction. He centered under the microscope a single red cell by a laser microbeam, and observed the arrival of white cells, whose number was increasing in time. Laser is light, and this means that lipid peroxidation might have developed in the centered cell. So, I decided to test the chemotactic power of the aldehydes. My young collaborator Marina Curzio was sent to Paris, where she used mostly the Boyden chamber method to measure chemotaxis. So, she was able to show that 4-hydroxynonenal and related aldehydes, but not the non-4-hydroxylated unsaturated and saturated aldehydes, are chemoattractant for rat neutrophils, whereas they promote random movements (chemokinesis) with human neutrophils. Hermann Esterbauer became very interested in this problem, and discussed with Marina and myself the single steps of the experiments. It became clear quickly that the aldehydes are rapidly destroyed by white cells, as well as by a lot of cells of different types. Ten minutes after the addition, only about 10% of the aldehyde was still present in the medium.

The destruction is due to a lot of different enzymes, as well as to the direct interaction of the aldehyde with –SH and NH_2-groups. How can the aldehyde signal reach far targets without being destroyed on the way? Hermann suggested to us to study the reactions of 4-hydroxynonenal with bovine serum albumin. He considered at first that probably the aldehyde might become bound to the protein by covalent stable bonds. Some enzyme might therefore be able to set the aldehyde free from the bond near to the far targets.

In Turin, however, we found that at least a portion of the aldehyde binds in a softer way. In fact, when the adduct is incubated in the absence of external aldehyde, a certain portion of the bound 4-hydroxynonenal is released in the medium: Hermann who was rather rigid in his

principles, did not trust that this was possible, but finally was convinced and accepted. This result was important, as it can show how 4-hydroxynonenal is transported by the blood to reach far targets, escaping from destruction.[13–16]

Another important point was to establish if the target cells have specific receptors for the active aldehydes. Preliminary experiments had shown that white cells preincubated with 4-hydroxynonenal do not respond a second time to the same aldehyde, whereas they still respond to the bacterial tripeptide. Moreover, cells pretreated with the last chemoattractant do not respond a second time to it, but still respond to 4-hydroxynonenal. This experiment showed that, if there are leukocytes receptors for 4-hydroxynonenal, these are different from those for the bacterial tripeptide. The real presence of receptors for the aldehyde in leukocytes was proven only when we succeeded to have tritiated 4-hydroxynonenal. By using this substance, we saw that it binds to some components of the cytosol, and not to membranes. The binding is specific, and reveals the presence of a medium affinity receptor. This cannot act through –SH groups, as the binding was shown in a medium saturated by the SH-reagent N-ethyl-maleimide. At this point, we were convinced that 4-hydroxynonenal and related aldehydes behave like a signalling agent in physiological conditions.

In order to prove this physiological activity, Hermann measured 4-hydroxynonenal content from a lot of tissues and cells, and found that concentrations similar to those shown to be active in signalling were present in the so called "normal" tissues. Malonaldehyde and 4-hydroxynonenal are normally present in the blood, mostly as bound to albumins and to lipoproteins.[18–25]

This means that a certain level of lipid peroxidation must occur in normal subjects. The lipid peroxidation enzymatic system of the endoplasmic reticulum might be responsible for this. Moreover, it is certain that long-living cells undergo a continuous remodelling of their subcellular organelles. The old structures become segregated inside autophagic vacuoles, where they undergo lipid peroxidation. Moreover, it is well known that 4-hydroxynonenal is present in exudates, as well as in the inflammatory areas.[26–28]

In 1984, Steinbrecher *et al.*[18] found that endothelial cell provoke lipid peroxidation in lipoproteins. Hermann became himself fascinated by a possible involvement of lipid peroxidation and 4-hydroxynonenal in the pathogenetic mechanisms of atherosclerosis and concentrated his attention especially on this topic.[19–25] Today, the increased internalization in endothelial cells and in macrophages of lipoproteins modified by 4-hydroxynonenal is considered to be a key point in understanding the mechanism of the formation of atheromas.

In the meantime, we had found in Turin another target of the signalling activity of 4-hydroxynonenal: phospholipase C, both in liver and in leukocyte membranes.[29–32] In this case, maximal stimulating activity was observed at a concentration of 0.1 μM, that was 10 times lower than that of maximal activity in the case of adenylate cyclase. This difference in active concentrations may explain the apparently intriguing results shown with adenylate cyclase and with phospholipase C. In fact, cAMP, the product of adenylate cyclase action, mostly acts on protein kinases A, whereas phospholipase C-mediated hydrolysis produces diacylglycerol, that is known to be a stimulator of the protein kinases C pathway. With liver cells, the integrated effects of 4-hydroxynonenal result in a moderate stimulation of protein kinase C activity.[33]

Another proof of the action of the aldehyde on hepatocytes is the discharge of Ca^{2+} from intracellular stores, found with 1 μM hydroxynonenal in a medium deprived of external Calcium.[34] It is known that IP_3, the other product of phospholipase C-mediated hydrolysis, produces the same effect.

Lipid peroxidation is low in tumours. Both in Graz and in Turin it had been shown that

addition of the aldehydes to tumor cells results in a decline of proliferation. We decided to go deeper on this point, by using especially two human leukemic cell lines, i.e. K562 erythroleukemic cells and HL-60 myeloblastic-promyelocytic leukemic cells. Both these cell types display a great expression of the oncogene c-myc. Moreover, both cell lines are very undifferentiated: K562 cells are unable to express the gene for γ-globin, whereas the HL-60 cells do not express the chemiluminescent specific neutrophil granules. The addition of 1 μM 4-hydroxynonenal to the cultures provokes in both cases a consistent decrease of the expression of c-myc (even c-myb in the case of HL-60).[39] Moreover, the expression of the gene for γ-globin reappears in K562 cells, whereas the HL-60 cells undergo a differentiation along the polymorphonuclear leukocyte line, as shown both by some recovery in the phagocytic activity, and by the appearance of chemiluminescent granules. Moreover, the marker antigens of polymorphonuclear leukocytes do appear.[35–39]

So, the aldehydes not only decrease cell proliferation, but also display a differentiating activity. In both cases, there is a delay in cell cycle, with great increase of cells in the G_0–G_1 phase, without apoptosis. As the promotion from G_1 to S phase in the cycle is controlled by cyclins (especially D_1 and D_2, a certain role being also covered by A), we investigated the behaviour of cyclin expression in HL-60 cells. We found that the expression of the mRNAs in these cyclins (but not for other cyclins) strongly decreased in the presence of 4-hydroxynonenal. It is noteworthy that in all studied cases, about 90% of added aldehyde disappears within 10 min, whereas the observed effects appear after 1–4 h, when no trace of the aldehyde can be detected. The effects of 1 μM aldehyde are transient (all the usual expressions are recovered within 5–6 h) but they can be prolonged for few days when the treatments of the cells with the aldehyde are systematically repeated.

Another target of 4-hydroxynonenal signalling has been found to be the heat shock factors in hepatocytes. In fact, 1 μM 4-hydroxynonenal provokes the expression of a subset of heat shock proteins.[41,42] This effect seems to be related to the activation of the heat shock transcription factor.[43]

Another line of experiences has shown an important signalling effect of 4-hydroxynonenal on human hepatic stellate cells (Ito cells). With such cells, 1 μM 4-hydroxynonenal is able to produce the expression of the gene for α-1[1] procollagen, as well as of its protein product. At the same time, the aldehyde provokes an increased expression of c-jun (but not of c-phos), as well as of its transcription factor AP-1. By confocal microscopy, it has been found that 4-hydroxynonenal, that is detected by a specific antibody obtained against an aldehyde-histidine adduct, prepared by Hermann Esterbauer, becomes quickly concentrated inside the nucleus, where at least three new proteins appear. Two of them have been identified as jun-kinases 1 and 2. It is noteworthy that Ito cells are thought to play an important role in fibrogenesis and in cirrhosis, and that chronic CCl_4-treatment provokes cirrhosis, that can be partially prevented by supplementation with high doses of vitamin E.[44–51]

Modulation of gene expression by HNE at low μM concentrations has been recently started for aldose reductase in rat smooth muscle cells [52] and for the fibrogenic cytokine transforming growth factor β1 (TGFβ1) in both murine and human macrophagic cell lines.[53]

In conclusion, we can state that, 4-hydroxynonenal, a toxic substance produced during lipid peroxidation, is not only a toxic substance, but even, at low concentrations existing in "normal" cells, a messenger for cell signalling (Table II). The described targets are numerous, but this happens also in the case of other signals. The nature of the response depends largely upon the nature of the target. What is necessary now is to discover the intimate mechanism of the reaction of the signal with the targets. Moreover, it is still unclear if the aldehyde reacts directly with the DNA bases, or if its effect is only indirect. It is a

TABLE II Actions displayed by 4-hydroxynonenal at low concentrations (usually 1 μM or less)

1. Strong activation of plasmamembrane adenylate cyclase in hepatocytes.
2. Stimulation of chemotaxis in rat polymorphonuclear leukocytes and of chemokinesis in human neutrophils.
3. Strong stimulation of phospholipase C in hepatocytes and polymorphonuclear leukocytes membranes.
4. Modest stimulation of hepatocyte protein kinase C.
5. Discharge of Ca^{2+} influx from the suspension medium into hepatocytes.
6. Block in the expression of the oncogene c-myc both in K562 erythroleukemic and HL-60 myeloblastic-promyelocytic leukemic cells.
7. Block of the expression of the oncogene c-myb in HL-60 cells. No effects on the Ras oncogene series.
8. Reexpression of γ-globin gene and of its protein product in K562 cells.
9. Maturation of specific chemiluminescent granules in HL-60; differentiation towards the granulocytic line.
10. Stimulation of the expression of heat shock protein in hepatocytes, by activation of the heat shock protein transcription factor.
11. Block of the expression of mRNAs for cyclins D_1, D_2 and A. Accumulation of cells in G_0–G_1 phase without apoptosis in HL-60 cells.
12. Stimulation of the expression of mRNA for procollagen α-1[I] and of its protein product in human hepatic stellate cells (Ito cells).
13. Stimulation of the expression of the oncogene c-Jun and of its transcription factor AP-1 in Ito cells.
14. Concentration of added 4-hydroxynonenal in the nucleus of Ito cell and contemporary appearance in the nucleus of jun-kinases 1 and 2.
15. Stimulation of the expression of the gene for aldose reductase in rat smooth muscle cells (5 μM HNE).
16. Up regulation of TGFβ_1 expression and synthesis in cells of the macrophage lineage (10 μM HNE).

pity that Hermann, whose discussions have been very stimulating on the progress in research, is not alive. We will continue, however, on the same pathway.

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