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Review Paper

The fundamental contribution of prof. Stirpe (and his research group) to the broadening of the scientific perspective on xanthine oxidoreductase

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ABSTRACT

Human xanthine oxidoreductase (XOR) is a multilevel regulated enzyme, which has many physiological functions, but which is also involved in several pathological processes. The contributions of Stirpe and his research group at the University of Bologna, to the development of knowledge on the XOR enzyme and its implications in physiological and pathological processes made a breakthrough in this field. Furthermore, their pioneering results paved the way for many research lines that are still actual and that have relevant clinical implications.

1. Biographical notes

Fiorenzo Stirpe was born in Castro dei Volsci (Frosinone, Italy) on January 28, 1932. His father, a physician, instilled in him the interest for biomedical sciences; such interest grew during high school, leading Stirpe to enroll in the Faculty of Medicine and Surgery at "La Sapienza", University of Rome. During this period, Stirpe left the intention of dedicating to surgery and he developed the idea to devote himself to the scientific research. In 1955, he graduated at the age of 23 years and his dissertation thesis about the ascorbic acid-induced oxidation was awarded as the best biological chemistry thesis of the year [1]. After graduation, he worked as a volunteer assistant at the Institute of Organic Chemistry of the University of Rome. Afterwards, he took the role of Lecturer in General Pathology at the University of Messina from 1957 to 1960. In those years, Stirpe also had experiences abroad; he worked at the Toxicology Unit of the Medical Research Council in Carshalton (University of Oxford), and later at the National Institutes of Health, in Bethesda (MD, United States). After his return to Italy, Stirpe continued his academic career taking the role of Senior Lecturer at the University of Siena in 1961. In 1964, Stirpe started to work at the Institute of General Pathology of the University of Bologna, where he spent the rest of his academic life, taking the role of Full Professor in 1970. The studies about xanthine oxidase (XO) of those years led to the discovery that this enzyme is actually a NAD^+-dependent dehydrogenase, which can be converted into oxidase. This discovery allowed other scientists to found out that in particular conditions oxidase may be responsible to damage the organism, thus opening a research field that is still ongoing today. In the early 1970s, Stirpe undertook another research line, which led to the identification, purification and characterization of new plant toxins, which he called "ribosome inactivating proteins" or RIPs. In 1984, he received the "Antonio Feltrinelli" Medicine Prize by the Accademia Nazionale dei Lincei and in 1995 he awarded the Pierce Prize from the 4th International Symposium on Immunotoxins, in Myrtle Beach, SC. In 1998, for his prestigious career, Professor Stirpe received the Honoris Causa Degree in Biological Sciences at the Second University of Naples, and in 2006 he received the title of Professor Emeritus from the University of Bologna.

2. The fructose-induced hyperuricemia and the fructose tolerance test

Since the beginning of his scientific career, Stirpe was interested in the metabolism of the purines and the mechanisms responsible for hyperuricemia that cause gout. In the wake of the publications of Perheentupa and Raivio [2] and Giordano et al. [3], he investigated in particular the fructose-induced hyperuricemia that depends on the unlimited phosphorylation of fructose at the expense of the ATP with the consequent formation of AMP, which is degraded up to xanthine, then transformed into uric acid by xanthine oxidoreductase (XOR) (Fig. 1). Stirpe developed a fructose tolerance test, which showed that the oral administration of fructose is able to cause the increase in uricemia and that this increase is more intense and more prolonged in gouty patients as well as in their apparently healthy children than in normal subject. Although in the absence of any symptom, the offspring of these patients showed an intermediate slope between that of healthy and sick subjects in the temporal course of this test [4].

For many years Stirpe helped gouty subjects, who had shown a pathological response to the oral fructose load test, modifying their diet

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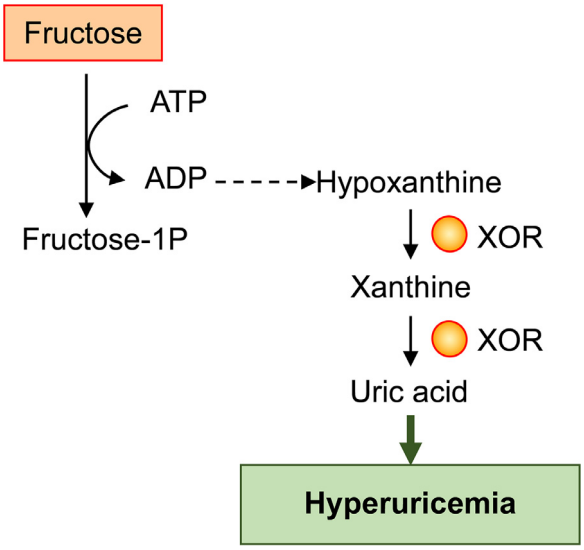

Fig. 1. Fructose-induced hyperuricemia. The unlimited phosphorylation of fructose, at the expense of the ATP, generates ADP and AMP, which are degraded up to hypoxanthine, then transformed into uric acid by xanthine oxidoreductase (XOR).

through a strong reduction in the consumption of sweet food, fresh fruit and alcoholic beverages. Although these patients had a meat-rich diet and interrupted the treatment with allopurinol, they experienced a normalization of uricemia, a strong reduction or a complete disappearance of pain and an improvement in the joint functions, allowing them to avoid the assumption of colchicine. The results of this dietetic control of the uricemia aroused great gratitude for Prof. Stirpe in patients, but this evidence was considered not sufficient by The Lancet, which, refusing the publication of the obtained data, discouraged the continuation of this line of research. The only other manuscript published on this type of diet to cure gout was authored many years later by a Stirpe collaborator [5].

The role of the fructose consumption in the onset of hyperuricemia and its consequences was subsequently rediscovered [6] and revised by Johnson and collaborators [7,8]. Since hyperuricemia and gout can also have other causes, the fructose tolerance test proposed by Stirpe could be very useful to identify patients who can benefit from a low fructose and alcohol-free diet, thus avoiding taking drugs, but it has never been introduced for this purpose in medical practice.

3. The discovery that even the xanthine oxidoreductase of mammals is a dehydrogenase

The XOR that catalyzes the formation of uric acid in mammals, including humans, was known as XO, whereas in all other living beings this enzyme was called xanthine dehydrogenase (XDH), on the bases of the electron acceptor used by the enzyme. The first studies that Stirpe published on this topic concerned the influence of the diet on the level of XOR activity in the liver of birds and rats [9–12].

Then Stirpe started to study the regulation of XOR from rat liver, observing that freshly prepared rat liver supernatant oxidizes xanthine more rapidly with NAD^+ than with O_2 as electron acceptor, and the storage at -20 °C of supernatant greatly increased the enzyme activity measured with O_2 [13]. A similar XO-activating effect was obtained through a proteolytic treatment of the liver supernatant that abolished the formation of NADH, whereas the enzyme activity in the presence of O_2 reached that observed with NAD^+ [14]. Analogous results were obtained: (i) by incubating at 37 °C the liver homogenate either in aerobiosis or under anaerobic conditions before separating the supernatant, (ii) by obtaining the supernatant after ultrasonic disruption of the homogenate, or (iii) by treating the supernatant at 37 °C with proteolytic enzymes or solvents. These results suggested that the rat liver enzyme is mostly a dehydrogenase that can be converted into an oxidase by the above treatments [15]. The same hypothesis was formulated about the human hepatic enzyme, which behaved as dehydrogenase when it was analyzed immediately and as oxidase after storage at -20 °C for 24 hours or after incubation at 37 °C for 5 min in the presence of xanthine either in anaerobiosis or with trypsin [16].

The XDH from rat liver extract was converted into XO by a thermolabile factor present in particulate subcellular fractions. This transition is not due to a proteolytic process, because it can be reverted by dithioerythritol and could be attribute to an enzyme oxidizing thiol groups [17]. The inhibitory effect of the NADH on hepatic XDH from both the chicken and the rat, but not on XO from rat liver together with the fact that the NAD^+ does not compete with the xanthine suggested that NAD+ and xanthine bind at different sites on hepatic XDH of both the chicken and rat. In addition, NADH was competitive for NAD^+ suggesting that both enzymes have two different sites: one for the dehydrogenation of xanthine and the other for hydrogen to be transferred to NAD^+ and then to the final acceptor [18]. These hypotheses were confirmed over thirty years later by the structural studies of the enzymatic molecule [19].

The fact that also the mammal's XOR is in origin a dehydrogenase was definitively demonstrated by the subsequent experiments published in 1972. This discovery opened the door to decades of research about a possible role for XOR in the pathogenesis of the tissue damage observed after ischemia/reperfusion, which may be related to the ability of the enzyme to produce the superoxide anion following the theory first proposed by Granger [20].

Most of the enzyme from rat liver supernatant behaves as NAD^+-dependent dehydrogenase that can be converted into oxidase by several treatments, as reported in previous articles. In most cases this conversion can be reverted by dithioerythritol. However, the XDH of bird liver cannot be transformed into oxidase. In the supernatant of the other organs of the rat the enzyme appears as an oxidase that can be reverted to dehydrogenase by dithioerythritol, but in the case of intestine. In addition, these results have shown that in mammals XOR is present in two interconvertible forms: XDH and XO and that XDH can be transformed into XO either irreversibly by partial proteolysis or reversibly through the oxidation of its thiol groups [21]. Noteworthy, the authors supposed the existence of an intermediate form of XOR able of using both NAD^+ and O_2 as the electron acceptor, as well as the inactivation of functionally crucial thiol group(s) during the transition from XDH to XO, which were demonstrated a lot after [22].

By assuming that the irreversibility of the intestinal XO was due to the action of proteolytic enzymes during the preparation of the supernatant, the latter was prepared in the presence of trypsin inhibitor and 2-mercaptoethanol, obtaining an intestinal XO of the rat reversible to XDH by incubation with dithioerythritol. These results indicated that in all the rat organs XOR is in origin a dehydrogenase and the conditions of enzyme preparation are responsible of its oxidase activity [23]. The XOR was purified by cow's milk without the traditional proteolytic treatment and consequently a reversible XO was obtained, confirming once again that both the XDH and the XO of mammals are the two faces of the same enzyme [24].

4. The xanthine oxidase-antibodies conjugates

In the last century, Ehrlich's "magic bullets" began to become a viable idea for many researchers around the world. Several types of hybrid molecules were synthesized linking a carrier, able to bind to specific cell membrane receptors, to a toxic payload, capable to kill the cell. In 1980, Stirpe's team supposed that type 1 RIPs could be good toxic moieties to be selectively delivered by a specific carrier to eliminate unwanted cell populations, such as cancer cells. The development of monoclonal antibody technology allowed the production of chimeric proteins named

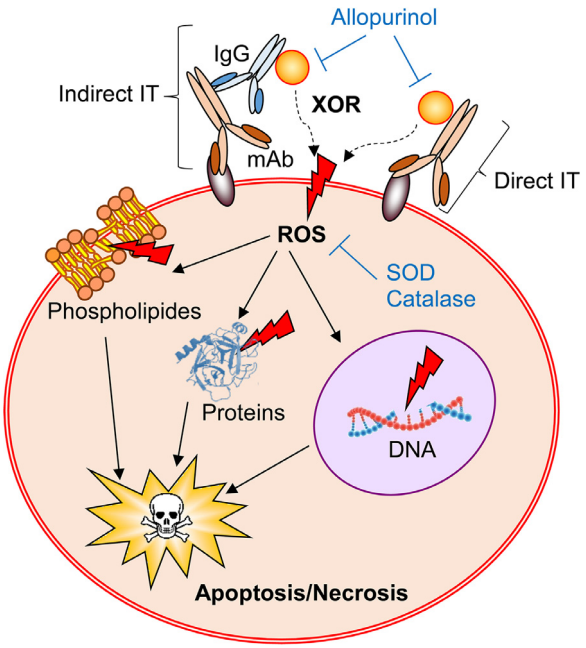

Fig. 2. Xanthine oxidoreductase (XOR) conjugates and cytotoxic effects. Selective cell killing was obtained by conjugating XOR to a monoclonal antibody (mAb) or to an anti-mouse IgG linked to a specific mAb. Both the direct and indirect approach led to specifically deliver reactive oxygen species (ROS) to target unwanted cells. Mechanisms of ROS cytotoxicity: ROS induce peroxidation of membrane lipids, DNA damage, and protein oxidation and lead to cell death, mainly via apoptosis through impaired mitochondrial function. Cytotoxic effects can be abolished by the XOR inhibitor allopurinol or by the ROS scavenging enzymes superoxide dismutase and catalase.

immunotoxins (ITs). Stirpe began to study the possibility to deliver toxic payloads on target cells starting the preparation of immunotoxins (see Chapter 6). In this perspective, XOR was conjugated to antibodies for experimental therapies.

The cytotoxicity of reactive oxygen species (ROS) produced by XO was exploited with the purpose of ex-vivo bone marrow purging for autologous grafting in multiple myeloma patients. Commercial XO was covalently linked to the monoclonal antibodies 8A and 62B1 recognizing human plasma cell-associated antigens. The conjugates were harmless to non-target cells and myeloid staminal cells, while retaining both immunological and enzymatic properties, and obtained a significant degree of target cell lysis, which was abolished by the XOR inhibitor allopurinol or by the ROS scavenging enzymes superoxide dismutase and catalase (Fig. 2) [25,26]. The selective cytotoxicity of XO-62B1 conjugate was potentiated by adding chelated iron, while no toxicity to mice was observed when it was i.v. injected [27].

XO-containing immunoconjugates were also prepared with an anti-CD3 monoclonal antibody recognizing T lymphocytes to avoid the graft-versus-host reaction through the ex vivo purging of allogenic transplants from T lymphocytes [28]. The death mechanisms triggered by XO-containing immunoconjugates to human peripheral lymphocytes was analyzed. XO-derived ROS induced both apoptosis and necrosis mostly dependent on the enzyme concentration [29]. However, at very low concentrations, the XO-derived ROS had a stimulating effect on human lymphocytes and 3T3 Swiss fibroblasts treated with mitogen factors [30].

XO-derived ROS were more toxic to B lymphoma-derived Raji cells than to peripheral human lymphocytes. Hydrogen peroxide and not superoxide ion was responsible for cell death that mostly was for apoptosis. The same death mechanism occurred when the lymphoma-derived Raji cells were selectively targeted either directly with an 8A-XO conjugate, or indirectly with an 8A mAb (murine) plus an anti-mouse IgG-XO conjugate (Fig. 2). XO-conjugates induced apoptosis of Raji cells with both direct and indirect methods in a dose-dependent manner [31].

5. The xanthine oxidoreductase in experimental and clinical pathology

In the meantime, as the world literature was enriching with research on the involvement of XOR in various pathological situations, at the University of Bologna another aspect of the XOR was being studied by Stirpe: the XOR potential as a damage marker.

The effect of hypoxia and ethanol on rat liver was studied: in isolated rat hepatocytes, the hypoxia gradually caused death and at the same time the leakage of XOR outside the cell, phenomena that were preceded by the transformation of the enzyme from XDH to XO and were accelerated by the presence of ethanol. In any case, the XO was reversible to XDH if incubated with dithioerythritol. Even rat liver XDH after incubation with human plasma or serum was converted into the reversible form of XO [28].

The systemic injection of kainic acid in the rat caused excitotoxic lesions of the olfactory cortex and induced an increase of the XO/XDH ratio after 4 and 48 hours and also an increase of both XDH and XO activities after 48-72 hours in the same area but not in hippocampus. The initial conversion of XDH to XO overlapped the appearance of seizures and appeared as a consequence of the local cell injury. The later increase of the total XOR activity overlapped with the morphological alterations of the damaged area showing necrosis and inflammatory changes of the cellular composition [32].

The XOR activities were determined in rat liver and serum samples at 24, 48 or 72 hours after intoxication with lethal doses of ricin, saporin or a saporin containing immuno-conjugate. All the treatments caused liver necrosis, however the death of sinusoidal cells preceded the necrosis of the hepatocytes in ricin treated animals, whilst in the liver of rats treated with free or conjugated saporin no damage was observed in sinusoidal cells. Ricin treatment induced a progressive conversion of the liver enzyme from XDH to XO, whilst it did not cause changes in the serum enzyme. There were no alterations of the liver enzyme in the rats treated with free or conjugated saporin, while these treatments induced a higher activity of serum XO. These differences were considered to depend on the formation of clots in the hepatic sinusoids, which prevented the release of the liver XOR in circulation, but favored the hypoxia that induced the conversion of the XDH to XO, in the case of ricin intoxication [33].

The serum level of XO is barely detectable in healthy people, but it can increase in pathological conditions, especially in liver diseases, because most of the serum XO in humans comes from the physiological turnover of hepatocytes [34]. To determine the level of XO in the human serum, a highly sensitive two-step competitive Elisa was developed, after purifying human milk XO and producing polyclonal rabbit antibodies anti-XO. The test allowed to ascertain that the concentration of XO protein in the serum of 10 out of 11 patients with liver diseases was more elevated than in controls [35]. The test was also utilized to determine the serum level of XO in 64 patients with chronic liver disease, finding that serum XO was significantly higher in these patients than in control subjects. The level of serum XO was correlated to cholestasis, but not to evidence of liver necrosis [36].

The XOR activities have been assessed on surgical human liver samples from histologically pathological areas or adjacent normal tissue. Total XOR activity was significantly higher in virus-related cirrhosis than in virus-negative cirrhosis or in controls. No variations have been observed in the XO/XDH ratio. Hepatocellular carcinoma samples have a significantly lower level of XOR activity than controls [37].

Between 1957 and 2005 (Fig. 3), Stirpe published 27 works concerning the metabolism of the purines and the XOR, only one tenth of all its

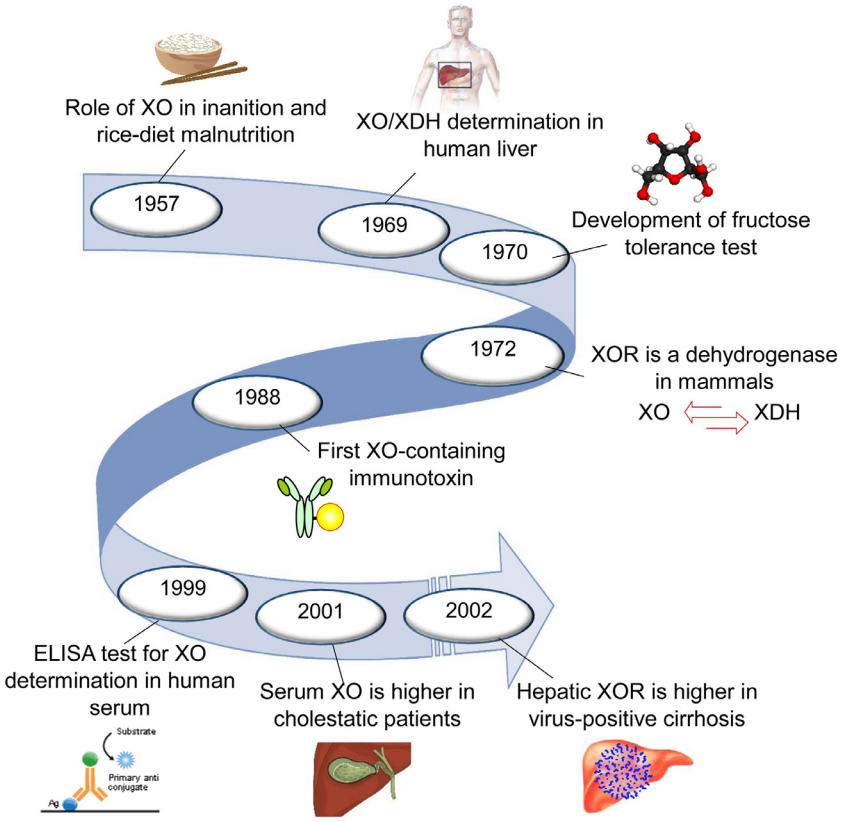

Fig. 3. The main milestones of xanthine oxidoreductase research carried out by Stirpe and co-workers.

production, but the imprint he left has given impulse to a huge number of publications in this field.

6. Plant toxins and immunotoxins

Beside to XOR, other important Stirpe's interests were related to plant toxins, many relevant publications focused on this topic and some of them represented important milestones on the matter. Stirpe's interest in ricin, a toxic protein from *Ricinus communis* [38], and other similar proteins will last throughout his career and profoundly influence the scientific community (reviewed in [39,40]).

In the early 1970s, in collaboration with other researchers from University of Bologna, he contributed with important results to the understanding of the mechanism of action of ricin. Later, Stirpe discovered that many other plant proteins, although having a much lower toxicity than ricin, had the same enzymatic activity as it. In 1982, he introduced for the first time the denomination "ribosome-inactivating protein" (RIP) to designate all the protein synthesis inhibitors able to inactivate the 60S ribosome subunit [41]. RIPs were classified into two types depending on the presence (type 2 RIPs) or the absence (type 1 RIPs) of a lectin chain, being some type 2 RIPs, as ricin, abrin and stenodactylin, very potent toxins. In 1990s, Stirpe's group observed that RIPs were able to depurinate a number of polynucleotide substrates, thus identifying for RIPs a wider enzymatic activity for which the name polynucleotide:adenosine glycosylase was proposed [42]. During his career Stirpe and his co-workers discovered about one hundred of new RIPs, amongst them some very toxic type 2 proteins from Adenia plants [43].

The first ITs containing type 1 RIPs were obtained conjugating different proteins to a monoclonal antibody against a T cells antigen (reviewed in [39,40]), contributing to the implementation of anticancer immunotherapy. Including the publications about XOR-containing ITs, Stirpe authored 66 articles describing conjugates and their possible medical applications. Most of the type 1 RIPs-containing ITs were produced against B or T-lymphocytes with the purpose of preventing the graft versus host disease in allogenic bone marrow transplantation or treating hematological malignancies, but ITs were also prepared and tested against solid tumors and for the nano-surgery of oculo-motility disorders. The most encouraging results were obtained in advanced multiple myeloma patients receiving autologous transplantation of IT-purged bone marrow [44,45] and in patients with advanced refractory Hodgkin's disease treated with an IT prepared by covalently linking saporin to an anti-CD30 monoclonal antibody [46].

In conclusion, also in the case of plant toxins, Stirpe contributed to large extent in a wide range of topics about RIPs from plants and related immunoconjugates. A great number of RIPs has been detected, purified and characterized by his research group and also a great number of ITs were developed and studied for their pharmacological potentialities.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Data Availability

No data was used for the research described in the article.

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