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# *Modified western blot technique in fast detection of heme oxygenase (HO-1/HO-2) in various tissues and organs of experimental animals*

The main role of heme oxygenase (HO; heme hydrogen-donor:oxygen oxidoreductase; EC 1.14.99.3) is to catalyze the NADPH and cytochrome P450 reductase-dependent oxidation of heme to biliverdin, iron and carbon monoxide (CO) (14). These by-products are of some great importance because of their multiple biological functions in living organisms. As is known, biliverdin is reduced to bilirubin through biliverdin reductase, and subsequently conjugated with glucuronic acid prior to its excretion in bile (9). Biliverdin and bilirubin scavenge reactive oxygen species and/or inhibit lipid peroxidation (3). The experimental and biochemical studies have shown that CO may be implicated in some physiological processes related with neurotransmission and/or neuromodulation of synaptic plasticity in the brain, similarly to nitric oxide (NO) (12). Moreover, this gaseous molecule is highly responsible for a homeostatic control of cardiovascular functions (4). In the last few years evidence has accumulated showing HO activity and its by-products as important factors in the cellular metabolism (11). The free iron, removed from the heme, is directly extruded outside the cell in order not to generate free radicals (6). To date, three isoforms of HO have been identified in mammalian tissues as follows: HO-1, HO-2, and HO-3.

HO-1, also known as the heat shock protein (HSP) is an inducible form of HO, hydrolyzing nearly 1% of heme in blood to equimolar quantity of CO and biliverdin, which is subsequently converted to bilirubin (1). It is believed that HSP protects cells exposed to non-physiological stress evoked by exposure to heavy metals, oxidizing stress, ultraviolet radiation and supra-physiological temperature (5). HO-1 is expressed in most tissues of living organisms. This enzyme consists of 289 amino acids (mouse/rat) or 288 amino acids (human) and its gene is located on chromosome 22, having ~ 88% of interspecies homology. Molecular mass of HO-1 is ranging between 31–33 kDa.

HO-2 is a non-inducible form of enzyme widely expressed in high concentrations within the brain (13). Low activity of HO-2 has been found in most tissues except for spleen. HO-2 comprises 315 amino acids (mouse/rat) or 316 amino acids (human). At the amino acid level, HO-1 and HO-2 are homologous in 42%. Molecular mass of HO-2 is ~ 36 kDa. Additionally, it has been shown that HO-2 through a CO liberation regulates the neural transmission in the central nervous system (2).

HO-3 possesses a low catalytic activity and unclear physiological role (10). It has been detected in spleen, liver, thymus, prostate, heart, kidney, brain and testis. HO-3 comprises 290 amino acids (~ 36 kDa). The predicted amino acid sequence has 90% homology with HO-2. The main function of HO-3 is heme sensing or binding rather than its degrading.

To date, the detection of HO isoforms in various tissues and organs of living organisms has been performed by using a western blot technique according to procedures described by Dwyer and coworkers (5). It should be stressed that there are no methods more eligible and sensitive enough to

quantitatively and/or qualitatively determine various proteins in tissues and organs of experimental animals. Nonetheless, this method is a time consuming technique because of an overnight (16–18 hours) electro-transfer of the investigational proteins onto specific membranes (5). The purpose of the present study was to elaborate and determine the most optimal conditions required for a quick detection of HO-proteins with western blot technique.

## MATERIAL AND METHODS

Materials. All chemical reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA) and ICN Biomedical Research Products (Irvine, CA, USA), whereas the apparatus for electrophoresis and western blotting was purchased in Bio-Rad (Hercules, CA, USA).

Animals. The experiments were carried out on a male albino Swiss mouse and a Wistar rat. The animals were kept under standardized laboratory conditions with free access to food (chow pellets) and tap water on natural light-dark cycle for seven days prior to the experiments. The mouse (weighing 25 g) and the rat (weighing 250 g) were killed by a cervical dislocation and the organs (liver, heart, lung, spleen, kidney, testis, and brain) were removed and immediately frozen, storing the organs at -60°C into the deep freezer (Angelantoni, Masa Martana, Italy).

Western blotting. Mouse and rat tissues were homogenized with the ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 1 μg/ml aprotinin, 100 μg/ml PMSF (phenyl-methyl-sulphonyl fluoride), 100 mM NaCl. Next, the samples of tissue homogenates were centrifuged in 4°C at 12,000 x g. After the precipitated and solubilized fraction was discarded, the protein concentration was determined in supernatant using a Chiron Diagnostics Total Protein Kit (Emeryville, CA, USA), based on biuret reaction according to Gornall et al. (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli et al. (8). Before the loading on the gel, samples were boiled for 7 min in the presence of 2 x SDS loading buffer (6.25 mM Tris-HCl at pH 6.8; 10% glycerol, 2% SDS, 640 mM β-mercaptoethanol, 0.01% bromophenol blue). Each sample containing the same quantity of total proteins (50 μg) was separated onto a 12% SDS polyacrylamide gel for 3 hours at 90 mA (5% stacking gel), and 60 mA (12% separating gel) by using an original Bio-Rad apparatus. A sample of 10 μl of the Kaleidoscope Prestained Standards (Bio-Rad) as a molecular marker was used. After electrophoresis, the gel containing separated proteins was incubated in the TGM buffer (25 mM Tris, 192 mM glycine, 15% methanol) for 20 min and subsequently, proteins were electro-blotted onto supported 0.45 μm nitrocellulose membrane (Bio-Rad) for 1 h. at 220 mA in the presence of the electro-blotting buffer (25 mM Tris, 20% methanol, 40 mM glycine). Afterwards, a membrane containing proteins was incubated in TBS buffer (138 mM NaCl, 2 mM KCl, 24 mM Tris, 5% nonfat milk) for 20 min. and Rabbit anti-Heme-Oxygenase-1 Polyclonal antibodies (Stressgen Biotechnologies, Victoria, Canada) diluted 1:5000 were added. These antibodies detect a 32kDa protein corresponding to the apparent molecular mass of HO-1, but can also cross-interact with HO-2. Incubating time with the primary antibody was reduced to 2 h. After this period, the membrane was extensively washed with TBS buffer three times for 10 min. and incubated with a Goat Anti-Rabbit Immunoglobulins (IgG) conjugated to alkaline phosphatase (DAKO A/S, Glostrup, Denmark) diluted 1:2000; for 30 min. Further, the membrane was washed twice for 5 min. and incubated in alkaline phosphatase buffer AP (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl pH 9.5) for 5 min. The last step was the incubation with BCIP/NBT mix (Sigma Aldrich, St. Louis, MO, USA), 20 ml per 10 cm x 10 cm membrane for 5 min. Adding 1M EDTA the reaction was terminated. Every intermediate step of immunoblotting was conducted at room temperature with gently rolling.

## RESULTS AND DISCUSSION

Immunoreactive HO-1 and HO-2 proteins were detectable in homogenates of organs by using Western blot technique. Our results indicate that the examined proteins could be detected in a very short time. Two hours of incubation of a nitrocellulose membrane with primary antibodies HO-1 was enough to detect HO. In contrast, some authors employed the procedures of detection of HO-1/HO-2 after 16–18 hours of electro-transferring (overnight) onto specific membranes (5). In the present study, the highest level of HO-1 was detected in the spleen of the experimental mouse and rat (Fig. 1 and 2). Additionally, the marked bands for HO-2 were observed in the rat liver and testis, whereas both isoforms were detected in the rat thymus and brain (Fig. 1). Similarly, the highest amount of HO-1 was detected in the spleen and liver of the examined mouse, although, the net bands were also observed for the kidney and thymus in the mouse (Fig. 2).

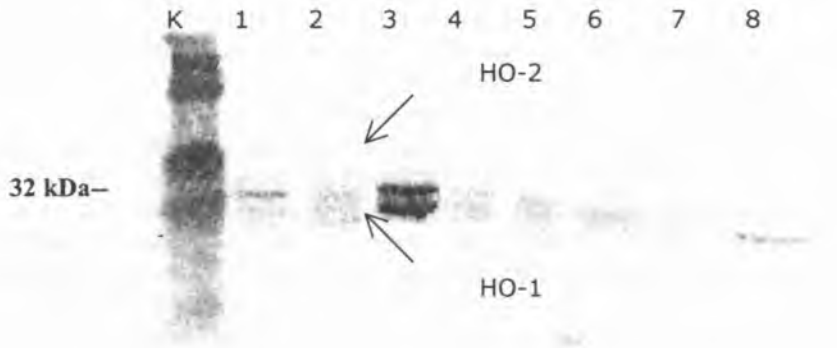


Fig. 1. Immunoblot illustrating both isoforms of HO-1 and HO-2 detected in various rat tissues and organs; K – kaleidoscope, 1 – liver, 2 – lung, 3 – spleen, 4 – thymus, 5 – brain, 6 – kidney, 7 – cerebellum, 8 – testis

The upper bands consecutively numbered from 1 to 8 reflect to HO-2, whereas the bottom bands are specific for HO-1

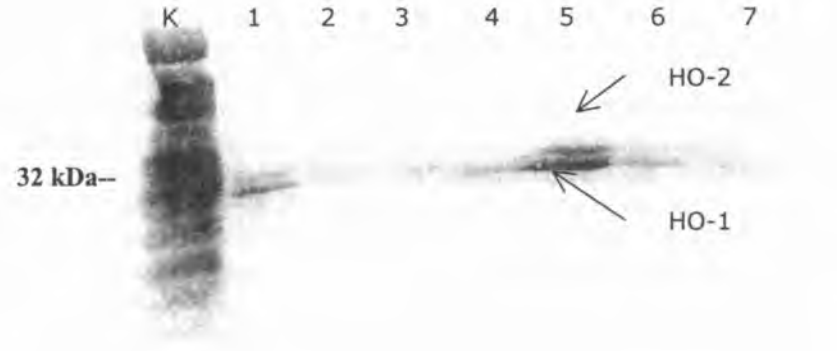


Fig. 2. Western immunoblot displaying two isoforms of HO-1 and HO-2 in mouse tissues; K – kaleidoscope, 1 – liver, 2 – lung, 3 – heart, 4 – kidney, 5 – spleen, 6 – thymus, 7 – brain

With our modification, the time required for detection of HO-1 and HO-2 was drastically reduced, since the time of electro-transferring onto nitrocellulose membranes has been shortened to 2 hours. It has to be clearly stated that the present study, which was a pilot experiment, allowed us to elaborate a methodology for further more advanced studies dealing with the detection of proteins involved in the stress oxidation processes during the inflammatory response, without additional apprehension about the loss of sensitivity to detect the requested proteins. Moreover, it was found that HO-1 and HO-2 are present in various organs of experimental animals, which may be potentially applicable in further advanced studies. Methodology, elaborated and presented herein permits the qualitative estimation of HO in tissues. Summing up, our methodology for detecting HO-proteins in tissues is scientifically valuable due to the reduction of time and high detectable selectivity to HO-1 and HO-2 proteins.

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SUMMARY

The present study was aimed at determining all indispensable conditions required to detect heme oxygenase (HO) with western blot technique. Our modified immunoblotting method allowed the detection of HO after 2 hours of electro-transferring onto nitrocellulose membranes that evidently shortened the time of research study without any loss of sensitivity and specificity to detect HO in various tissues and organs of experimental animals. HO was detected in the brain, heart, kidney, liver, lung, spleen, testis, and thymus of the examined mouse and rat, in a quantity for providing evidence that this modified immunoblotting technique is sensitive enough to elicit the existence of this enzyme in various animals' tissues and organs. In conclusion, our modified western blot method permits the fast detection of HO that may be useful in further more advanced quantitative studies.

Zmodyfikowana technika western blotu w szybkim wykrywaniu oksygenazy hemowej (HO-1/HO-2) w różnych tkankach i organach zwierząt doświadczalnych

Celem pracy było wyznaczenie niezbędnych warunków wymaganych do wykrycia oksygenazy hemowej (HO) techniką western blotu. Modyfikacja metody immunoblottingu pozwoliła na wykrycie HO po 2 godz. transferu na nitrocelulozowe membrany, co ewidentnie skróciło czas doświadczeń bez utraty czułości i swoistości dla HO w różnych tkankach i narządach badanych zwierząt. HO była wykryta w mózgu, sercu, nerkach, wątrobie, płucach, śledzionie, jądrach i grasicy myszy i szczura, w ilości potrzebnej do zapewnienia, że zmodyfikowana metoda immunoblottingu jest wystarczająco wrażliwa na ujawnienie istnienia tego enzymu w różnych tkankach i organach u zwierząt. Zmodyfikowana metoda western blotu pozwala na szybką detekcję HO, co może być korzystne dla przyszłych bardziej zaawansowanych badań ilościowych.