Cholesterol decreases secretion of the secreted form of amyloid precursor protein by interfering with glycosylation in the protein secretory pathway

Josè Luis GALBETE, Teresa RODRIGUEZ-MARTIN, Elisa PERESSINI, Piergiorgio MODENA, Roberto BIANCHI and Gianluigi FORLONI

Laboratory of Biology of Neurodegenerative Disorders, Istituto di Ricerche Farmacologiche 'Mario Negri', 20157 Milano, Italy

INTRODUCTION

β-Amyloid (βA) is the major component of senile plaques and is deposited in cortical and meningeal blood vessels of the brain in Alzheimer’s disease (AD). These features, together with tangles and cortical atrophy, are the neuropathological hallmarks of AD. βA derives from a larger transmembrane glycoprotein, the amyloid precursor protein (APP), with multiple isoforms generated by differential splicing of a gene that maps to human chromosome 21 and is highly conserved across the species [1,2]. Genetic and biochemical studies indicate a central role of βA in the pathogenesis of AD. Linkage studies have shown an association between APP gene mutations and some cases of familial early-onset AD [3], another genetic background associated with AD; mutated forms of presenilin 1 and 2 and the allele e4 of apolipoprotein E resulted in the increased production of βA.

Neurotoxic effects of synthetic peptides with similar amino acid sequences as βA and its fragments have been described, and neurodegenerative transgenic mice carrying mutated human APP [4] or βA [5] have been bred. APP is subject to N- and O-glycosylation and undergoes a series of proteolytic cleavages that lead to the release of βA or of a non-amyloidogenic secreted form of APP (APPs). We used primary neuronal and glial cultures to investigate how cholesterol affects the production and secretion of APPs. Exposure to cholesterol for 2 h did not change the neuronal release of APPs; after 6 h APPs release was slightly lower, whereas 24 h of exposure decreased APPs in the medium by approx. 60%.

The time courses were similar in astrocytes and microglia pre-treated with cholesterol for 6 h. We investigated the influence of cholesterol on the physiological secretion of APPs in primary cultures: neurons, microglia and astrocytes. We determined the total APP expression and secretion after exposure to cholesterol, and the influence of cholesterol on the APP secretion pathway including the insertion of APPs into the plasma membrane.

Key words: β-amyloid, Alzheimer’s disease, primary tissue cultures.

**Cerebral deposits of β-amyloid (βA)** are a major feature in Alzheimer’s disease. βA is derived from amyloid precursor protein (APP). APP is subject to N- and O-glycosylation and undergoes a series of proteolytic cleavages that lead to the release of βA or of a non-amyloidogenic secreted form of APP (APPs). We used primary neuronal and glial cultures to investigate how cholesterol affects the production and secretion of APPs. Exposure to cholesterol for 2 h did not change the neuronal release of APPs; after 6 h APPs release was slightly lower, whereas 24 h of exposure decreased APPs in the medium by approx. 60%.

The time courses were similar in astrocytes and microglia pre-treated with cholesterol for 6 h. We investigated the influence of cholesterol on the physiological secretion of APPs in primary cultures: neurons, microglia and astrocytes. We determined the total APP expression and secretion after exposure to cholesterol, and the influence of cholesterol on the APP secretion pathway including the insertion of APPs into the plasma membrane.
membrane rigidification on the inhibition of APPs secretion by cholesterol. At the cellular level, we also investigated the influence of cholesterol on APP glycosylation.

MATERIALS AND METHODS

Materials

The monoclonal anti-APP antibody 22C11, protease inhibitor cocktail tablets and the glycosidases N- and O-glycosidase were obtained from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibody 6E10 was from Senetek PCL (St Louis, MO, U.S.A.). Monoclonal anti-biotin antibody conjugated with agarose, anti-mouse IgG antibody conjugated with peroxidase, water-soluble β-cyclodextrin-encapsulated cholesterol, monensin, brefeldin A, tunicamycin and neuraminidase were from Sigma (St Louis, MO, U.S.A.). Sulphosuccinimidobiotin was purchased from Pierce (Rockford, IL, U.S.A.). The enhanced chemiluminescence (ECL) detection kit was from Amersham (Little Chalfont, Bucks., U.K.). Nitrocellulose was from Schleicher and Schuell (Dassel, Germany). Protein G–Sepharose was obtained from Pharmacia Biotech.

Cell cultures

For neuron cultures, brain tissue was removed from fetal rats at embryonic day 17. Cortical cells were dissociated in serum-free medium containing 0.1% trypsin (Difco) and 25 μg/ml deoxyribonuclease for 5 min at room temperature, plated in 24-well dishes (Falcon) precoated with poly-(l-lysine) (50 μg/ml) (Sigma) at 4 x 10⁶ cells per dish in Eagle’s minimal essential medium (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM glutamine, then cultured at 37°C in a water-saturated air/CO₂ (19:1) atmosphere. After 4 days in vitro, non-neuronal cell division was halted by exposure to 10 μM cytosine arabinoside. The neuronal cell cultures are characterized elsewhere [17].

Astrocytes and microglial cells were prepared from newborn rats or mice, as described previously [18]. Cells were grown in poly-(l-lysine)-coated Primaria (Falcon) dishes containing Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% (v/v) FCS and 2% mM glutamine. Cultures were kept at 37°C in a humidified air/CO₂ (19:1) atmosphere. The medium was changed every 2 days. Astrocytes were harvested from primary mixed cultures by the method of Yao et al. [19]. After 10 days of culture, flasks containing mixed glial cultures were given fresh medium and placed on a shaker at 37°C for 12–16 h. After 30–60 min the medium was aspirated and replaced with fresh DMEM. The adherent cells (astrocytes) were then exposed for 5 min to 0.25% trypsin, followed by the addition of an equal volume of DMEM/10% (v/v) FCS. The suspension was centrifuged and the pellet was resuspended in medium containing 10% (v/v) FCS. Cells were plated in 24-well dishes. The application of the complex of methyl-β-cyclodextrin and cholesterol as well as the other treatments with the various drugs were performed after 7 days of culturing of cells in a serum-free medium.

Northern blot analysis

Total cellular RNA was isolated according to the acid guanidinium/phenol/chloroform method [20]. RNA was separated on 1.2% agarose/formaldehyde gels and transferred to Nylon 66 filters (Gene Screen Plus; Dupont). The total APP mRNA probe was a 1.0 kb EcoRI fragment of a mouse cDNA clone representing the βA and proximal 3'-untranslocated portions of the APP mRNA. cDNA probes were labelled with a randomly primed DNA labelling kit from Amersham and [³²P]dCTP. The labelled probes were purified through a Sephadex 650 column (Pharmacia). The membranes were hybridized to ³²P-labelled specific probes. After exposure of blots to X-ray films at −80°C for the appropriate duration, densitometric analysis of autoradiograms was performed with an IBAS 2 image analyser (Zeiss) [21]. β-Actin mRNA was used as an internal standard to normalize the expression of APP.

Gel electrophoresis and immunoblotting

Cells were lysed in SDS/PAGE sample buffer. The cell lysates were centrifuged for 5 min at 10000 g and the supernatants were boiled at 95°C. Extracellular medium was centrifuged at 15000 g for 15 min to remove the cellular debris. Proteins were precipitated in 50% (w/v) trichloroacetic acid and sodium deoxycholate (2 mg/ml), washed with acetone and solubilized in sample buffer, as described previously [22]. Equal amounts of protein were separated on 7.5% (w/v) polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose. Membranes were blocked by incubation in 5% (w/v) non-fat dried milk in PBS for 1 h at room temperature. After being washed four times in PBS containing 0.1% (v/v) Tween 20 for 30 min, membranes were incubated with primary antibody diluted in blocking buffer (1:100) for 1 h at room temperature. After a further wash, the membranes were incubated with secondary antibody diluted in blocking buffer (1:500 anti-mouse IgG peroxidase conjugate; Sigma) for 1 h at room temperature, washed again four times for 25 min in PBS containing 0.1% Tween 20, then detected with the enhanced chemiluminescence system. The densitometric analysis of bands was calculated with an IBAS 2 image analyser (Zeiss).

Immunoprecipitation was performed with monoclonal antibodies 6E10 and 22C11. Antibody 6E10 recognizes an epitope between residues 1 and 17 of βA sequence [23] and was used to precipitate x-APPs. Antibody 22C11 recognizes a region between residues 66 and 81 of APP [24]. In brief, after harvesting, samples of medium supplemented with a protease inhibitor cocktail were centrifuged for 1 min at 1000 g to remove non-adherent cells and then at 15000 g for 10 min to remove other cell debris. Samples were preadsorbed on Protein G–Sepharose for 30 min at 4°C and immunoprecipitated overnight at 4°C in the presence of specific antibody and Protein G–Sepharose. Precipitates were washed four times with PBS, boiled in Laemmli sample buffer and separated by SDS/PAGE [7.5% (w/v) gel].

Digestion with endoglycosidase and treatment with tunicamycin

After removal of the culture medium, the cells were washed with PBS and lysed in PBS/0.5% (v/v) Triton-100 for 30 min at 4°C in the presence of an EDTA-free protease inhibitor cocktail. Detergent-insoluble proteins were removed by centrifugation. Cell extracts (20 μl) were incubated at 37°C for 18 h with 10 m-units/ml N- or O-glycosidase and 0.1 m-unit/ml neuraminidase. To control for non-specific degradation, parallel samples were incubated in the absence of enzymes. Samples were resuspended in 2× Laemmli buffer, then boiled and analysed by SDS/PAGE [25]. Tunicamycin, which prevents the N-glycosylation of glycoproteins, was added directly to the neurons overnight.

Cell-surface biotinylation and detection of biotinylated APP

Cortical neurons (10⁴, 5 days old) were cell-surface-biotinylated by incubation with 0.4 mg/ml sulphosuccinimidobiotin as described [26]. The reaction was stopped by washing the cells twice with Hapes buffer. Cells were harvested and lysed. Biotinylated
proteins were precipitated with monoclonal anti-biotin–agarose; samples were boiled in 25 μl of sample buffer. Immunoprecipitates were subjected to SDS/PAGE [7.5 % (w/v) gel] and electrotransferred to nitrocellulose. Biotinylated APP was detected with monoclonal antibody 22C11 by enhanced chemiluminescence detection.

RESULTS

We evaluated the effect of cholesterol on the metabolism of APP by measuring APPs by immunoblotting in the media of rat neuronal, microglial and astroglial primary cultures. To favour the delivery of cholesterol to the cells, the compound was applied to the cultures in a complex with methyl-β-cyclodextrin, as described previously [14]. Figure 1(A) illustrates the dose–response pattern of the effect of cholesterol on APPs in neurons after 24 h of exposure. APPs progressively decreased with cholesterol dosage, from 40 % at 50 μg/ml to 70 % at 1 mg/ml; at the latter concentration, neuronal viability was affected. A similar dose–response pattern occurred in astrocyte medium (Figure 1B), although at 50 μg/ml the decrease did not reach statistical significance.

Exposure of microglia to cholesterol markedly reduced APPs (80 %) in the medium, starting from 50 μg/ml cholesterol (Figure 1B). At this dosage the effect was already at its plateau. We used 50 μg/ml cholesterol for subsequent studies, because this concentration was close to the physiological amount of free cholesterol in interstitial fluid [27].

The time course of the effect of cholesterol (50 μg/ml) on APPs secretion in neuronal, microglial and astroglial cultures is shown in Figure 2. In the control condition the amount of APPs in the medium of neuronal cultures rose progressively with time. When the neurons were exposed to cholesterol, APPs in the medium started to decrease from 6 h; however, this became statistically significant only after 24 h of exposure (control 100 ± 3.3 %; cholesterol 35.2 ± 4.1 %; Figure 2A). The levels of APPs in the medium of astrocytes and microglia after 2 and 6 h of exposure to cholesterol did not differ from controls, but in both conditions APPs secretion was significantly decreased after 24 h (Figures 2B and 2C). However, in the microglial cultures...
Time (h)

Figure 3 Effect of cholesterol on APP mRNA expression in neurons and glial cells

Neuronal (A), astroglial (B) and microglial (C) cells were exposed to cholesterol (50 μg/ml) for different durations in a serum-free medium. Cholesterol was complexed with cyclodextrin. Total RNA was extracted from the cells and APP mRNA was determined by Northern blot analysis; β-actin mRNA was measured as an internal standard. The autoradiographic signal was quantified by densitometric analysis (OD, attenuation) and normalized to the β-actin mRNA signal. Results are means ± S.E.M. for four determinations. *P < 0.01 compared with control group (Dunnett’s test).

Figure 4 Effect of GM-1 on APPs secretion in neurons and glial cells

Neuronal, microglial and astroglial cells were exposed to the ganglioside GM-1 (100 μg/ml) for 24 h in a serum-free medium. APPs was determined in the culture medium by immunoblotting; the bands were quantified by densitometric analysis and are expressed as percentages of the controls. Results are means ± S.E.M. for four or five determinations.

this inhibition was similar to that in the neurons (65%), whereas in the astrocytes the effect was less intense (25%; Figure 1B). To verify the influence of cholesterol on APP synthesis we used Northern blot analysis to determine the expression of APP mRNA after exposure of the cells to cholesterol for different periods (Figure 3). Cholesterol significantly decreased APP mRNA levels after 2 h of exposure in all three cell types. However, at 6 and 24 h the APP mRNA in the cells was similar to that in controls, except for the 24 h treatment in astrocytes, in which APP mRNA was significantly increased (Figure 3B).

Because cholesterol decreases membrane fluidity [28], it has been hypothesized that in other cell types [14,15] the inhibition of APPs secretion by cholesterol was due to membrane rigidification. We investigated this possibility by using two substances that strongly decrease membrane fluidity. First, the cells were treated with GM-1 (100 μg/ml), a ganglioside that efficiently decreases the membrane microviscosity [29], for 24 h; APPs was measured in the culture media. No significant change in APPs secretion was observed in the media of the cells (Figure 4). Similar results were obtained with prion protein fragment PrP 106–126, which also induces a marked decrease in cellular membrane microviscosity in glial and neuronal cells [30] (results not shown). It is therefore unlikely that the inhibition of APPs secretion induced by cholesterol is a consequence of membrane rigidification.

To evaluate the effect of cholesterol on total APP production we determined the intraneuronal content of APP by immunoblot, in comparison with APPs secreted into the medium. Figure 5(A) shows examples of immunoblots from media and neurons exposed to cholesterol for different durations. As in Figure 2(A), in the control medium the level of APPs increased progressively with time. No significant difference was observed between the cholesterol-treated cultures at 2 and 6 h whereas at 24 h the APPs signal was much weaker than in control or vehicle-treated (methyl-β-cyclodextrin) cells. Three bands characterized neuronal APP: the lower band, at approx. 100 kDa, was identified as the immature form of the protein; the other two, at 110 and 125 kDa, were the mature forms. After 2 h of exposure to cholesterol, the neuronal APP pattern was not altered but at 6 h the higher-molecular-mass bands decreased and by 24 h they had almost completely disappeared.

There was a significant decrease in APPs with brefeldin A (10 μg/ml) and monesin (10 μM), two treatments that prevent the proteins from maturing in the Golgi complex and endoplasmic reticulum [31,32]. With these treatments the bands for the mature forms of neuronal APP were strongly decreased after 16 h exposure. These results were obtained with the antibody 22C11, which does not distinguish between α- and β-secretase product [24]; therefore to verify the separate contributions of α- and β- APPs under our conditions, we used a specific antibody, 6E10, that recognized only the α-secretase product [23]. The media of neurons treated with vehicle or cholesterol (50 μg/ml) for 24 h were immunoprecipitated with 6E10, to separate the α-APPs, and then with antibody 22C11 to identify the β-APPs. An example of immunoblots with these preparations is shown in Figure 5(B): cholesterol strongly decreased the α-APPs signal
Cholesterol affects amyloid precursor protein metabolism

Figure 5 APP immunoblots of medium and cellular extracts from neurons

(A) Neuronal cells were exposed to cholesterol (50 μg/ml), β-cyclodextrin (1 mg/ml), brefeldin A (10 μM) and monensin (10 μM) for different durations in a serum-free medium. Cell lysates (lower panel) and media (upper panel) were analysed by immunoblotting and APP was determined with antibody 22C11. A clear decrease in APPs (108 kDa) was evident in the medium of cells treated with cholesterol for 24 h and with brefeldin A and monensin for 16 h whereas β-cyclodextrin did not change the secretion of APPs in comparison with the control. In the cell lysates, three bands corresponding to the different maturation states of APP (100, 110 and 125 kDa) appeared in the control. At 6 h the treatment with cholesterol decreased, and at 24 h almost abolished, the signals corresponding to the glycosylated forms of APP, similar results were obtained with brefeldin A and monensin. (B) Immunoblots from media of neurons exposed for 24 h to cholesterol (50 μg/ml) immunoprecipitated with antibody 6E10, which recognizes only α-APPs. A subsequent immunoprecipitation was performed with antibody 22C11 to determine the β-APPs form in the cell media. Cholesterol strongly decreased the α-APPs signal, whereas β-APPs was only partly attenuated. The positions of molecular mass markers are indicated at the left.

but only slightly attenuated the β-APPs band. Thus the decrease in APPs induced by cholesterol was due almost completely to the α-secretase metabolism. Because APP is a highly glycosylated membrane glycoprotein containing both N- and O-glycans, we evaluated the influence of digestion with N- and O-glycosidase, and the influence of neuraminidase, for comparison of these results with the effect of cholesterol. An example of an immunoblot from neuronal lysate treated for 18 h with neuraminidase (0.1 unit/ml), N-glycosidase (N-g, 10 m-units/ml) and/or O-glycosidase (O-g, 10 m-units/ml) is shown in Figure 6(A). Neuraminidase completely abolished the higher-molecular-mass APP band, whereas there was an increase in the signal intensity of the 110 kDa band. A different APP pattern was induced by N-glycanase, which also abolished the 125 kDa band but increased the intensity of the APP at the lowest molecular mass. An APP pattern similar to that observed with cholesterol treatment of the cells was found with the combination of neuraminidase and O-glycanase, which induced a complete shift of the APP signals to a single intense band at the lower molecular mass. As expected, the treatment with O-glycanase alone did not change the pattern of APP because its activity was inhibited by the presence of sialic acid groups. In addition, the treatment of the neurons overnight with tunicamycin, a classical inhibitor of N-glycosylation, induced an APP pattern similar to that observed with the cell lysate treated with N-glycase (Figure 6B). Thus the post-translational modification of APP under our conditions was essentially due to the glycosylation of the protein that was inhibited by cholesterol exposure.

The effect of cholesterol on the ratio between the signals of mature and immature APP in neuronal cells is quantified in Figure 7. After 2 h of exposure the ratio was unchanged, but

© 2000 Biochemical Society
after 6 and 24 h it decreased markedly. Similar results were obtained in astrocytes (results not shown). Thus the inhibition of APPs secretion by cholesterol seemed to be the consequence of an intracellular effect on APP maturation.

To verify whether the immature form of APP reached the membrane compartment, we measured APP inside the membrane by using the protein biotinylation technique. As shown in Figure 8, biotinylation of cellular surface proteins identified the APP associated with the membrane. In the control condition the two post-translationally modified forms of APP reached the membrane, whereas only the immature form was found in the cholesterol-treated neurons. Interestingly, after treatment with brefeldin or monensin, no more mature APP reached the membrane but a small amount of immature APP was detected.

**DISCUSSION**

The influence of cholesterol on APP metabolism and βA production has been investigated in various experimental models [14–16,33–35]. Here we have shown that in primary cultures of neurons, astrocytes and microglia, exposure to cholesterol markedly decreased APPs, this regulation appearing mainly at the post-translational level.

Low concentrations of cholesterol significantly decreased the secretion of APPs, with an identical time course in all three cell types. Similar results were obtained by Racchi et al. [15] in COS cells; they showed a 75% decrease in APPs secretion within 2 h of exposure to low doses of cholesterol. The inhibition of APPs production was also observed by Bodovitz and Klein [14] in an HEK 293 cell line stably transfected with APP 770, although in that case extremely high cholesterol concentrations were used (0.6–2.4 mg/ml). These authors proposed that the inhibition of APPs secretion by cholesterol was due to a change in membrane fluidity that decreased the interaction of APP with APP and consequently the β-secretase cleavage. At variance with previous data [15], in the primary cultures cholesterol had a significant effect on APPs secretion only after 24 h of exposure and in the range of concentrations (50–100 μg/ml) found in human interstitial fluid [27].

To verify the influence of membrane fluidity on α-secretase cleavage we measured APPs secretion after exposing the cells to substances that induce membrane rigidification. PrP 106–126 and GM1, both of which induce robust membrane rigidification, did not alter the APPs secretion. Our data therefore indicate that the influence of cholesterol on α-secretase cleavage is unlikely to be a consequence of changes in cell membrane fluidity. In agreement with this, cholesterol’s effect on APPs was significant only after 24 h exposure, whereas the alteration of membrane fluidity was seen immediately. We used antibody 22C11, which recognizes the N-terminal portion of APP [24] and does not distinguish between the α- and β-secretase products. However, by using a different, specific, antibody we determined α- and β-APPs separately; the influence of cholesterol was essentially on α-secretase metabolism.

We found that cholesterol inhibited the APP mRNA expression in all three cell types, although the effect was restricted to the first 2 h of exposure, which might partly account for the decrease in APPs production. However, because the protein has a short half-life (20–30 min), the consequence of the decrease in mRNA levels on APP synthesis is presumably small, as confirmed by an immunoblotting analysis of total APP, which showed a slight decrease.

APP maturation occurs through the Golgi complex and endoplasmic reticulum, where APP is N- and O-glycosylated [7], as confirmed under our conditions with the digestion of APP with neuraminidase, N-glycase and O-glycase and the treatment with tunicamycin. By immunoblot analysis of the immature and mature forms of APP in the neurons (and astrocytes) after exposure to cholesterol, we have shown that cholesterol progressively abolished APP maturation within 24 h. The APP pattern after the exposure of the neurons to cholesterol was similar to that obtained by digestion with endoglycosidase; the decrease in APPs secretion by cholesterol is probably due to the effect on APP glycosylation. We used two substances to test this, monensin and brefeldin A, that act with different mechanisms on the Golgi complex and endoplasmic reticulum, interfering with protein maturation. Like cholesterol, both substances inhibited APP maturation and decreased APPs secretion.

These results therefore confirmed that primary post-translational modifications of APP might cause the decrease in APPs. This is particularly interesting because it has been demonstrated that in neuronal cells a large percentage of intracellular βA is produced from immature APP [36–38]. Furthermore, post-translational changes in APP studied in platelets have been correlated with the severity of AD [39].

The molecular mechanism implicated in this effect is not known but, from previous investigations [40,41], a direct interaction is possible between cholesterol and APP at the endoplasmic reticulum or Golgi complex. In neurons these organelles are the site of the generation of βA 1–40 and 1–42 [38]. Furthermore, we show that, unlike with monensin and brefeldin A, most of the neuronal immature APP after treatment with cholesterol reached the cell membrane. Thus the non-glycosylated form of APP is inserted into the membrane but is not available for α-secretase cleavage. This is consistent with the observation that the principal determinants of α-secretase cleavage seem to be the conformation around the cleavage site [42], which is influenced by the state of maturation of APP.

A decrease in APP secretion without changes in the cell-associated full-length APP was observed in the brain of APP transgenic mice treated with dietary cholesterol [33]. In contrast, Simons et al. [16] showed that cholesterol depletion decreased βA...
generation in hippocampal neurons infected with recombinant virus carrying APP. In this case the secretion of APPs was not modified. In the latter study, human APP processing was analysed in rat cells with only some hours of survival after infection [16]. Thus the genetic manipulation and the overexpression of APP might have caused some alterations in APP metabolism. Our results are based on a more ‘physiological’ approach, determining the endogenous neuronal APP processing affected by low concentrations of cholesterol. Under similar conditions, Racchi et al. [15], using non-neuronal cells, obtained results similar to ours.

In conclusion, we have shown that cholesterol decreased the secretion of APPs in glial and neuronal cells. In neurons this effect was associated with a slight decrease in APP mRNA expression and a substantial effect on protein maturation. As a consequence of exposure to cholesterol, a large amount of immature APP reached the cell membrane but was not available for cleavage, decreasing the production of APPs. Thus cholesterol affects APP processing and potentially increases the amyloidogenic pathway producing Aβ peptide. This mechanism might involve cholesterol in the pathogenesis of AD, explaining the clinical evidence that high levels of cholesterol are also a risk factor in non-vascular dementia.

This work was partly supported by the Italian Ministry of Scientific Research (P. N. R. Farmaci 4/1.3.3.2.1).

REFERENCES