Prothrombin Synthesis and Degradation in Rat Hepatoma (H-35) Cells: Effects of Warfarin

By Pumin Zhang and J.W. Suttie

Vitamin K is a substrate for the enzyme catalyzing the carboxylation of specific glutamyl residues to γ-carboxyglutamyl residues in hepatic precursors of a limited number of plasma proteins, including prothrombin. The γ-carboxylation of these proteins can be blocked by the anticoagulant warfarin; and in the bovine and human, warfarin treatment results in the secretion of under-γ-carboxylated forms of prothrombin into plasma. In the rat, this response is not seen, but plasma prothrombin concentrations are drastically decreased. This response has now been studied in rat hepatoma (H-35) cells in which prothrombin secretion is decreased 90% by incubation in the presence of warfarin. Neither prothrombin mRNA levels nor the apparent rate of prothrombin message translation were decreased when cells were cultured in the presence of warfarin rather than of vitamin K. The pool of intracellular prothrombin precursors is increased threefold by warfarin treatment, and this pool is rapidly secreted when vitamin K is administered. In contrast, continued incubation in the presence of warfarin resulted in the degradation of 60% of this pool in 24 hours. When transport of secretory proteins to the golgi apparatus was blocked with Brefeldin A, this precursor pool was γ-carboxylated in the presence of vitamin K and no degradation occurred. Lysosomal enzyme inhibitors did not block the degradation, and the data suggest that, in rat hepatocytes, under-γ-carboxylated prothrombin is specifically targeted to a pathway of protein degradation located in the endoplasmic reticulum.

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MATERIALS AND METHODS

Cell culture. The H4IIEC2 strain of the H-35 rat hepatoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as monolayers in Dulbecco’s modified Eagle’s medium (DME; Sigma Chemicals, St Louis, MO) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). About 1 × 10^6 cells were inoculated into a T25 flask (Corning Laboratories, Corning, NY) with 5 mL of DME—FBS10. After 3 days of incubation at 37°C in humidified air containing 5% CO₂, cells were refed and cultured for 2 more days to confluence. Each experiment was conducted on confluent cells.

In experiments using 35S-labeling of proteins, confluent cells were incubated in 4 mL of DME—FBS10 containing 50 μCi/mL 35S-Met (DuPont NEN Research Products, Boston, MA) for overnight labeling or in 2 mL of Met-free DME containing 100 to 250 μCi/mL 35S-Met for pulse labeling.

Northern blot analysis. Vitamin K (10 μg/mL) or warfarin (1 μg/mL) was added to the culture medium of confluent cells and incubated for various times. Total RNA was isolated from these cells in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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cells as described in Chomczynski and Sacchi\(^\text{14}\) with minor modifications. The quality and quantity of RNA preparations were judged by measuring the absorbance of 260 nm and 280 nm. All preparations had a ratio of A\(_{260}\) over A\(_{280}\) of approximately 2.0. Total RNA (10 \(\mu\)g) from each sample was separated on a 1.2% agarose gel containing 2.2 \(\mu\)L mL of formaldehyde in the presence of ethidium bromide. The gel was electro-blotted onto a Nytran membrane (Schleicher and Schuell, Keene, NH) under conditions specified by the manufacturer of the membrane. The blots were washed briefly in 2 \(\times\) SSC (15 mmol/mL NaCl/15 mmol/mL sodium citrate, pH 7.0) and baked at 80°C for 1 hour.

A rat prothrombin partial cDNA (obtained from R. Bolger, University of Wisconsin-Madison) and a chicken \(\beta\)-actin cDNA (obtained from Dr. A.D. Atie, University of Wisconsin-Madison) were labeled to a specific activity greater than 1 \(\times\) 10\(^6\) cpmm/\(\mu\)g DNA with a random-primer labeling kit (Pharmacia, Piscataway, NJ). Labeled probe was separated from unincorporated \(^{32}\)P-nucleotide by a Nick-column (Pharmacia). RNA blots were hybridized to prothrombin probe in pipazine-N-N'-bis(2-ethanesulfonic acid) (PIPES) buffer (50 mmol/mL PIPES/100 mmol/mL NaCl/50 mmol/mL Na\(_2\)PO\(_4\); pH 6.8/1 mmol/mL EDTA/5% sodium dodecyl sulfate [SDS]) containing 1 \(\times\) 10\(^8\) probe/mL at 65°C overnight.\(^\text{20}\) The blots were washed twice in 5 \(\times\) SSC/5% SDS, once at room temperature and once at 65°C. The quantity of prothrombin probe bound was determined by scanning blots with a Blot Analyzer (Betagen Corp, Waltham, MA). The amount of prothrombin mRNA in a total RNA sample was expressed as the ratio of prothrombin probe bound (counts per minute [cpm]) to that of \(\beta\)-actin.

**immunoprecipitation analysis.** \(^{35}\)S-labeled cells were lysed with 0.5 mL of 0.5% Triton X-100/0.5% sodium deoxycholate/2 mmol/L EDTA/20 mmol/mL benzmamide/2 \(\mu\)g/mL aprotinin in phosphate-buffered saline (PBS; 137 mmol/mL NaCl/2.7 mmol/mL KCl/4.3 mmol/mL NaHPO\(_4\)/1.5 mmol/mL KH\(_2\)PO\(_4\); pH 7.4) and the cell lysates were centrifuged for 2 minutes in a desktop centrifuge to remove cell debris. Total incorporation of \(^{35}\)S-Met into cellular protein was measured by a determination of radioactivity precipitated by trichloroacetic acid.\(^\text{21}\) In pulse/chase experiments, total incorporation of \(^{35}\)S-Met into protein was not used for normalization because the total incorporation declined with time because of protein turnover. In these experiments, the sum of \(^{35}\)S-radioactivity present in the cell culture medium and in the cell lysates was quantitated by immunoprecipitation. About 0.1% of the total \(^{35}\)S-Met incorporated into protein in vitamin-K--sufficient cells during the incubation was found in prothrombin. As shown in Fig 1A, the amount of prothrombin secretion over a 24-hour period in the presence of warfarin was less than 10% of that secreted by cells grown in the presence of vitamin K. Intracellular accumulation of prothrombin precursors in warfarin-treated cells was nearly three times greater than in vitamin-K--treated cells. Warfarin treatment reduced the total (intracellular plus extracellular) prothrombin production in the cultures by 50%. Autoradiography of immunoprecipitates (Fig 1B) verified the specificity of the immunoprecipitation technique for prothrombin, and showed the same effect of warfarin on prothrombin production. Prothrombin secreted into the media in the presence of warfarin or vitamin K migrated at the same position as purified rat prothrombin, but intracellular prothrombin, as expected,\(^\text{20}\) exhibited a lower molecular weight.

**Effect of warfarin on prothrombin mRNA concentrations.** Because the large decrease in prothrombin production in the presence of warfarin could have resulted from a decrease in prothrombin gene expression, prothrombin mRNA was quantitated by Northern blot analysis. Total cellular RNA was sequentially blot hybridized to a prothrombin cDNA probe and then to a \(\beta\)-actin cDNA probe. The prothrombin mRNA in both warfarin and vitamin-K--treated cells migrated just above 18S rRNA at about 2 kb. Neither short-term (0.5 to 3 hours) nor long-term (12 to 48 hours) treatment of cells with 1 \(\mu\)g/mL warfarin altered the ratio of prothrombin/\(\beta\)-actin mRNA seen when cells were cultured in the presence of 10 \(\mu\)g/mL vitamin K (Table 1). The ratio of prothrombin to \(\beta\)-actin mRNA was also unaltered in the livers of vitamin-K--sufficient, vitamin-K--deficient, or warfarin-treated rats (data not shown). These data indicate that prothrombin mRNA levels in H-35 cells are unaltered by vitamin K status and that the decrease in prothrombin pro-
EFFECT OF WARFARIN ON PROTHROMBIN SYNTHESIS

Table 1. Effect of Warfarin on Prothrombin mRNA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Hours of Incubation</th>
<th>Vitamin K</th>
<th>Warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.5</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.4 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>B</td>
<td>12.0</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
<td>2.2 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>48.0</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.3</td>
</tr>
</tbody>
</table>

H-35 cells were incubated in 10 μg/mL vitamin K or 1 μg/mL warfarin. Values are quantitative Northern blots of prothrombin/β-actin ratios ± SD for four assays.

Production in warfarin-treated cultures is not caused by a decreased availability of prothrombin message.

Effect of warfarin on prothrombin synthesis rate. The rate of prothrombin synthesis, as measured by a 5-minute pulse of 35S-Met, was assessed to determine whether warfarin inhibition of prothrombin mRNA translation was responsible for decreased prothrombin production. Confluent cells were incubated in the presence of 10 μg/mL vitamin K or 1 μg/mL warfarin for 24 hours, washed, and labeled for 5 minutes with 100 μCi/mL of 35S-Met. No 35S-labeled prothrombin was secreted into the media during this 5-minute period. Newly synthesized prothrombin was immunoprecipitated from cell lysates and quantitated. Cells incubated in the presence of warfarin for 24 hours before the 35S-Met pulse had an apparent rate of prothrombin synthesis of 580 ± 40 cpm/10^6 cpm compared with 320 ± 20 cpm/10^6 cpm for cells previously incubated in the presence of vitamin K (mean ± SD for 6 assays). These data suggest that warfarin was increasing rather than decreasing the translation rate of prothrombin in RNA. Because neither the cellular content of prothrombin mRNA nor the rate of its translation appeared to be negatively influenced by warfarin, the decrease in prothrombin production seen in the presence of warfarin most likely involved the fate of the intracellular pool of prothrombin.

Effect of warfarin on degradation of the intracellular prothrombin precursor pool. Confluent cells were labeled with 35S-Met for 24 hours in the presence of warfarin to create a 35S-labeled pool of prothrombin precursors. The cell cultures were then washed to remove 35S-Met, and incubation was continued in the presence of either warfarin or vitamin K. Labeled prothrombin in both media and cell lysates was quantitated by immunoprecipitation. In vitamin-K-chased cells, the level of intracellular prothrombin precursors rapidly declined (Fig 2A, ○) and an equivalent increase in extracellular prothrombin (Fig 2A, □) was seen. However, in warfarin-chased cells, the level of prothrombin precursors decreased (Fig 2A, ●), without a corresponding increase in extracellular prothrombin (Fig 2A, ■). The total amount of 35S-labeled prothrombin (intracellular plus extracellular) in vitamin-K-chased cells remained unchanged (Fig 2B, ○) over 24 hours, indicating no degradation of the previously labeled pool. In contrast, there was a continuous decline in the total amount of labeled prothrombin in warfarin-chased cells (Fig 2B, ●). At the end of 12 hours, 50% of the initial
Fig 2. Effect of warfarin on degradation of intracellular prothrombin precursors. Confluent cells were labeled with 35S-Met (50 μCi/mL in DME-FBS10) in the presence of warfarin (1 μg/mL) for 24 hours. The labeled intracellular pool of prothrombin precursor was chased by incubation of the cells with vitamin K (25 μg/mL in DME-FBS10) or warfarin (1 μg/mL in DME-FBS10) for 24 hours. Prothrombin was quantitated by immunoprecipitation as described in Materials and Methods. (A) Changes in intracellular (●) and media (○) 35S-prothrombin in the presence of warfarin and intracellular (●) and media (○) 35S-prothrombin in the presence of vitamin K. (B) Changes in the total amount of 35S-prothrombin in the presence of warfarin (●) or vitamin K (○).

amount of 35S-prothrombin in the warfarin-chased cells had been degraded.

A characteristic property of the vitamin-K-dependent, γ-carboxyglutamyl-containing plasma clotting factors is their adsorption to insoluble heavy metal salts. When the culture media from vitamin-K-chased cells was treated with BaSO4, approximately 90% of the prothrombin was adsorbed and, by inference, was normally γ-carboxylated. In contrast, the small amount of prothrombin in the medium of warfarin-chased cells was not adsorbed by BaSO4 (Fig 3). The total amount of 35S-labeled protein secreted into the media, as measured by radioactivity in the washed trichloroacetic acid precipitate of culture media, was measured at 6, 12, and 24 hours. Total secreted protein did not differ in vitamin-K- or warfarin-chased cells (data not shown). Thus, the rapid excretion of prothrombin from vitamin-K-chased cells was not a result of a nonspecific effect of vitamin K on secretory proteins, but must be related to the vitamin-K-dependent specific γ-carboxylation of this intracellular pool.

Effect of the intracellular prothrombin pool size and degree of carboxylation on degradation. The observed degradation of prothrombin precursors in warfarin-treated cells could be a consequence of the extent of precursor accumulation, or a consequence of the undercarboxylation of this pool. To distinguish between these possibilities, the fate of an accumulated but fully carboxylated prothrombin precursor pool was studied. The antibiotic Brefeldin A (BFA), which blocks transport of proteins from the endoplasmic reticulum (ER) to the golgi apparatus, was used to obtain cells with an elevated pool of carboxylated prothrombin precursors. Cells were labeled with 35S-Met in the presence of warfarin as before. One hour before chasing, BFA was added into the cell culture medium. The cells were then washed and chased for 6 hours in the presence of BFA and either vitamin K or warfarin. As previously observed, about 10 times as much prothrombin was secreted to the media in the presence of

Fig 3. Effect of warfarin treatment on adsorption of prothrombin to BaSO4. Prothrombin in the media of cells incubated with vitamin K or warfarin for 6 hours (Fig 2) was adsorbed to BaSO4 as described in Materials and Methods. Prothrombin was quantitated by immunoprecipitation. Data are mean ± SD for six assays.
then were rapidly degraded. In contrast, the previously existing pool (labeled in the previous thrombin precursors (those labeled in the final 30 minutes) were not degraded until 6 hours after being synthesized and then were rapidly degraded. In contrast, the previously existing pool (labeled in the previous 24 hours) was continuously degraded. These data show that degradation of prothrombin precursors was not a random process, and, because less than 5% of the prothrombin was secreted before 6 hours (Fig 4B), the data suggest that the most recently synthesized precursors were in a pool protected from degradation. However, with time, the two pools apparently equilibrated because the final extent of degradation and secretion of newly synthesized precursors was essentially the same as that of the existing pool (Fig 4A).

A similar experiment to that described above was conducted, but cells were chased with 20 μg/mL vitamin K instead of warfarin. There was an initial delay of 30 to 45 minutes in the secretion of newly synthesized prothrombin that might reflect the time needed for complete posttranslational modifications other than the carboxylation event. However, by 3 hours, 90% of the radioactivity incorporated into prothrombin by either 24 hours of incubation or a 30-minute pulse had been secreted.

Effect of lysosomal inhibitors on the degradation of intracellular prothrombin precursors. To determine whether the intracellular prothrombin precursor pool is degraded by lysosomal action, the inhibitors leupeptin and NH4Cl, which have been shown to inhibit lysosomal protein degradation, were added into cell culture medium. Confluent cells were labeled with 35S-Met in the presence of warfarin for 24 hours, as previously described. Leupeptin or NH4Cl was added for 1 hour before and 6 hours after the 35S-Met was chased with cold Met. Continued incubation in the presence of warfarin, with no inhibitors added, resulted in a 49% loss of the intracellular prothrombin pool. In the presence of leupeptin or NH4Cl, 30% and 50% of the pool was lost (Table 4). The inability of NH4Cl or leupeptin addition to abolish the degradation of intracellular prothrombin in warfarin-treated cells suggested that lysosomal degradation is not the major cause of turnover of this pool.

**DISCUSSION**

These studies have shown that the rat hepatoma cell line used (H-35) is an appropriate model for studying the influence of vitamin K as compared with warfarin. However, the data in Table 2 show that prothrombin secretion even in the presence of vitamin K was effectively inhibited by BFA. In a subsequent experiment, it was shown (Table 3) that there was little degradation of the intracellular prothrombin precursor pool that accumulated in the presence of warfarin and was chased in the presence of vitamin K plus BFA. However, in cells in which incubation was continued in the presence of warfarin and BFA, nearly 40% of the prothrombin was degraded. The intracellular prothrombin remaining after 6 hours was treated with BaSO4 to adsorb γ-carboxylated prothrombin. Of the original prothrombin (326 ± 12 cpn/mL lysate) in the vitamin-K– and BFA-treated cells, less than 10% (31 ± 2 cpn/mL lysate) remained after BaSO4 adsorption. Prothrombin in the cells incubated with warfarin and BFA was 208 ± 7 cpn/mL lysate before BaSO4 adsorption and 208 ± 14 cpn/mL lysate after adsorption. The ability of BaSO4 treatment to adsorb 90% of the prothrombin precursor from cells treated with vitamin K and BFA, but none of the precursor pool from cells treated with warfarin and BFA, suggests that the process responsible for the degradation of prothrombin precursors is specific for the under-γ-carboxylated forms.

**Effect of time of synthesis on the degradation of prothrombin precursors.** Although the previous data clearly establish that the under-γ-carboxylated pool of prothrombin that accumulates in warfarin-treated cells is selectively degraded, they do not indicate whether the degradation is completely random or related to time-dependent events during protein processing. Confluent cells were incubated with warfarin and with or without 35S-Met for 24 hours, washed, and incubated for an additional 30 minutes in the presence of warfarin. During this period, the previously 35S-labeled cells were incubated with cold Met, and the previously unlabeled cells were incubated with 35S-Met. The cells were again washed and incubated in the presence of warfarin for up to 24 hours.

The data in Fig 4A show that the newly synthesized prothrombin precursors (those labeled in the final 30 minutes) were not degraded until 6 hours after being synthesized and then were rapidly degraded. In contrast, the previously existing pool (labeled in the previous 24 hours) was

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**Table 2. Effect of BFA on Prothrombin Secretion**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Medium Prothrombin (cpm/mL)</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Total (cpm)</th>
<th>Degraded (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin K</td>
<td>4,955.3 ± 164.5</td>
<td>24</td>
<td>22</td>
<td>4,707.8</td>
<td></td>
</tr>
<tr>
<td>Vitamin K + BFA</td>
<td>37.2 ± 7.4</td>
<td>12</td>
<td>13</td>
<td>24.3</td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>498.7 ± 29.0</td>
<td>20</td>
<td>20</td>
<td>228.7</td>
<td></td>
</tr>
<tr>
<td>Warfarin + BFA</td>
<td>74.2 ± 19.8</td>
<td>12</td>
<td>13</td>
<td>24.3</td>
<td></td>
</tr>
</tbody>
</table>

H-35 cells were labeled with 35S-Met (50 μCi/mL in DME–FBS10) in the presence of warfarin (1 μg/mL) for 24 hours. One hour before chasing, BFA (10 μg/mL) in MeOH (or MeOH) was added to the cell culture media. Cells were then washed and chased for 6 hours with vitamin K (25 μg/mL) or warfarin (1 μg/mL) plus BFA (10 μg/mL) or MeOH in DME–FBS10. After 6 hours, 35S-labeled prothrombin in cell culture media was quantitated by immunoprecipitation as described in Materials and Methods. Data are the means of four to six determinations ± SD.

**Table 3. Effect of Vitamin K Administration on Degradation of Prothrombin Precursors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Total (cpm)</th>
<th>Degraded (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before incubation</td>
<td>348.8 ± 20.9</td>
<td>1.8 ± 0.3</td>
<td>350.4</td>
<td></td>
</tr>
<tr>
<td>Vitamin K + BFA</td>
<td>362.2 ± 12.2</td>
<td>1.8 ± 0.3</td>
<td>363.0</td>
<td>6.3</td>
</tr>
<tr>
<td>(6 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warfarin + BFA</td>
<td>208.6 ± 6.6</td>
<td>2.5 ± 0.9</td>
<td>211.2</td>
<td>39.5</td>
</tr>
</tbody>
</table>

H-35 cells were labeled with 35M-Met (50 μCi/mL in DME–FBS10) in the presence of warfarin (1 μg/mL) for 24 hours. One hour before the chasing, 10 μg/mL BFA was added into cell culture media. The cells were washed and chased for 6 hours with vitamin K (25 μg/mL) or warfarin (1 μg/mL) plus BFA (10 μg/mL) in DME–FBS10. After 6 hours, 35S-labeled prothrombin in cell culture media and cell lysates was quantitated by immunoprecipitation as described in Materials and Methods. Data are the means of four to six determinations ± SD.
ence of the vitamin K antagonist on prothrombin synthesis. In both the intact rat and in the studies reported here, warfarin treatment results in a decreased secretion of prothrombin and in an accumulation of an intracellular prothrombin precursor pool. The decrease in prothrombin production in the H-35 cell line was shown not to be accompanied by a decrease in hepatic prothrombin mRNA, and these studies and previous studies in intact rats have shown that the prothrombin precursor pool is not static, but is actively turning over. The response of the rat to warfarin administration is, therefore, much different than in the human or bovine, where varying amounts of partially carboxylated prothrombin species are secreted to the plasma.

The data in Fig 2 clearly show that the fate of intracellular prothrombin in the warfarin-treated cells is degradation rather than secretion, and the lack of effect of leupeptin or NH₄Cl on this process (Table 4) suggests that it is a nonlysosomal process. The most likely candidate is, therefore, the endoplasmic reticulum-localized activity that has been shown to degrade a number of abnormal proteins or accumulated subunits of multimeric proteins. The signal for degradation is clearly related to the under-γ-carboxylation of this pool. The antibiotic BFA effectively blocked prothrombin secretion from the cell in the presence of warfarin or vitamin K, but degradation of the prothrombin pool occurred only in the presence of warfarin (Tables 2 and 3), where the majority of the accumulated pool was under-γ-carboxylated. However, some factor other than degree of carboxylation is involved in the degradation process. The data in Fig 4 show that the most recently synthesized prothrombin is not degraded for a significant period of time. This delay cannot represent movement of protein to the golgi, because degradation activity occurs when transport of prothrombin from the endoplasmic reticulum to golgi is blocked by BFA.

A recent report of prothrombin synthesis in a human (HepG2) hepatoma cell line suggested that the detergent used in the commercially available injectable form of vitamin K (aquamephyton; Merck, West Point, PA) stimulates prothrombin secretion from cultured human (HepG2) cells. This possible artifact could not have influenced these data because total protein secretion was not influenced by incubation in the presence of vitamin K or warfarin.

These studies raise the possibility that the under-γ-carboxylated prothrombin pool is targeted for degradation by

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**Table 4. Effect of Lysosome inhibitors on Degradation of Prothrombin Precursor**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Total Degraded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 h)</td>
<td>408.0 ± 23.6</td>
<td>—</td>
<td>409.0</td>
</tr>
<tr>
<td>Control (6 h)</td>
<td>220.8 ± 14.1</td>
<td>26.4 ± 0.7</td>
<td>247.2</td>
</tr>
<tr>
<td>100 μg/mL leupeptin (0 h)</td>
<td>411.1 ± 20.3</td>
<td>—</td>
<td>411.1</td>
</tr>
<tr>
<td>100 μg/mL leupeptin (6 h)</td>
<td>248.2 ± 31.5</td>
<td>30.1 ± 1.7</td>
<td>279.3</td>
</tr>
<tr>
<td>75 mmol/L NH₄Cl (0 h)</td>
<td>390.5 ± 23.0</td>
<td>—</td>
<td>390.5</td>
</tr>
<tr>
<td>75 mmol/L NH₄Cl (6 h)</td>
<td>186.1 ± 14.7</td>
<td>6.6 ± 0.4</td>
<td>192.7</td>
</tr>
</tbody>
</table>

H-35 cells were labeled with [³⁵S]-Met (50 μCi/mL in DME-FBS10) in the presence of warfarin (1 μg/mL) for 24 hours. One hour before chasing, leupeptin or NH₄Cl was added into cell culture media. Then cells were washed and chased for 6 hours with DME-FBS10 containing 1 μg/mL warfarin and leupeptin or NH₄Cl. Prothrombin in cell culture media and cell lysates was quantitated by immunoprecipitation as described in Materials and Methods. Data are the means of four to six assays ± SD.
interaction with another protein and that this association is decreased after carboxylation. It is not unlikely that a chaperone protein might be involved in the substrate/carboxylase complex and at least one such protein, hsp 78 or BIP, has been shown to be isolated with this complex.\(^\text{26}\) This protein, or a similar protein, might be involved in targeting the degradation process. Although these studies have provided an explanation for the lack of secretion of under-\(\gamma\)-carboxylated prothrombin in the warfarin-treated rat, they have not defined the difference between the rat and the bovine or human in this respect. Whether the signal for degradation lies with a species difference in the processing of prothrombin, or whether it lies in a difference between rat prothrombin and the prothrombin of other species, will require further investigation.

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Prothrombin synthesis and degradation in rat hepatoma (H-35) cells: effects of warfarin

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