Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 24-31

UBIQUITINATION OF THE RAT UTERINE ESTROGEN RECEPTOR: DEPENDENCE ON ESTRADIOL

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Received June 27, 1995

Using polyclonal antibodies against estrogen receptor and ubiquitin, the ubiquitination of the estrogen receptor has been demonstrated in both *in vitro* and *in vivo* conditions. The ubiquitination of the estrogen receptor is estradiol specific and is enhanced by estradiol. Estrogen withdrawal is associated with decreased ubiquitination of the estrogen receptor.

A novel mechanism for selective protein degradation was proposed by Hershko and Tomkins (1) which consisted of several essential components, most notably ubiquitin (2). Ciechanover *et al.* (3) demonstrated that several molecules of ubiquitin were conjugated to the proteins to be degraded in an ATP-dependent manner by isopeptide linkages to the ε-amino lysine of the protein substrate. Since ubiquitin protein conjugates were degraded rapidly with the release of free and reusable ubiquitin, a model was proposed according to which the conjugation of ubiquitin with protein is an obligatory event in protein breakdown (4). The C-terminus of ubiquitin is linked by an isopeptide bond to specific internal lysine residues of target proteins in a multistep process (4). Ubiquitin may be conjugated to the first ubiquitin, or to another lysine of the protein resulting in multiubiquitinated proteins (5).

The concentration of transcriptional regulators like the steroid receptors has to be regulated in the cell. How these receptors are timed and degraded is not known. The half life of the mammalian estrogen receptor (ER) in the absence of the hormone is ~5 days. In the presence of estradiol, the ER levels fall within an hour. The normal levels of the ER are reached within 4-6 hours that can be blocked by actinomycin D or cycloheximide (6). Thus, replenishment of receptors in the cytosol is contributed mainly by *de novo* synthesis of the receptors. Only 10% of the replenished receptors are contributed by recycling of the receptors that have moved out of the nucleus (7). Thus the ER has to be recognized and degraded selectively. The only known mechanism for selective degradation of proteins is the ubiquitin pathway. Therefore it was important to examine whether the ER was also degraded by mechanisms belonging to the ubiquitin pathway. For this purpose, it was important to identify ER-ubiquitin conjugates in the cell.

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0006-291X/95 $12.00

MATERIALS AND METHODS

Anti-ubiquitin antibody, 5-bromo 4-chloro 3-indolyl phosphate (BCIP), Estradiol 17β, diethylstilbestrol, testosterone, dexamethasone and progesterone, were obtained from Sigma Chemical Co. Nitrocellulose membranes were purchased from Schleicher and Schuell Inc., USA. Goat anti-rabbit IgG and alkaline phosphatase coupled goat anti-rabbit IgG were purchased from Genei, Bangalore. Phenylmethylsulfonyl fluoride (PMSF) and silver nitrate were purchased from E-Merck, Germany. Estradiol-bees-wax pellets were prepared by the addition of estradiol to molten bees-wax in the ratio of 1:1000. All the other chemicals used were of the analytical grade and were purchased from local commercial establishments.

Maintenance and surgery of rats: 3 months old female rats from an inbred colony of Wistar strain were used in the experimental studies. The animals were maintained under natural dark and light cycles (12 hours of light and 12 hours of darkness). Food (standard pellet food supplied by Hindustan Lever Ltd. India) and water were available *ad libitum* to the animals. Ovariectomy and subcutaneous implantations of estradiol: Bees wax (1:1000) pellets were done in rats under light ether anaesthesia.

Preparation of anti-estrogen receptor antibodies: The activated ER was purified to homogeneity from goat uterine cytosol following the procedure of Zafar and Thampan (8) and antibodies were raised against this protein in rabbits.

Ubiquitination of ER *in vitro*: Rats (3 months old) were ovariectomized under light ether anaesthesia and were sacrificed ten days after the surgery. The uteri of these rats were excised and the adjoining fat tissue was removed. The uteri were slit longitudinally and cut into small pieces. The uterine pieces were incubated in Dulbecco's minimal essential medium (DMEM) pH 7.6 containing 20 nM estradiol in a shaker water bath maintained at 37^{o}C for 0, 15, 30, 60, 120, 180 and 240 minutes. A control incubation of uteri at 37^{o}C for 240 minutes in the absence of estradiol was performed. At the end of the incubation, the uteri were homogenised in cold TEMN buffer (50 mM Tris-HCl pH 7.6, 1 mM EDTA, 12 mM Monothioglycerol, 50 mM NaCl and 0.2 mM PMSF). The uterine cytosol uteri was prepared following the procedure of Van der Hoeven (9). The concentration of the protein in the cytosol was estimated by the Lowry's method (10). Cytosol (1 mg protein) was incubated in triplicates with anti-ER IgG (1:50) overnight at 4^{o}C. To precipitate the antigen-antibody complex, the cytosol was incubated for an additional two hours with goat anti-rabbit IgG (1:200) at 4^{o}C. The immunoprecipitates were pelleted following centrifugation at 10,000 X g for 10 minutes and subjected to SDS-PAGE on 7.5% gels, in triplicates. One of the gels was stained with silver nitrate (Fig 1A) and the other two were transferred to nitrocellulose membranes. The nitrocellulose membranes were exposed overnight either to anti-ER antibody (Fig 1B) or to anti-ubiquitin antibody (Fig 1C). The nitrocellulose membranes were re-exposed to alkaline phosphatase-coupled anti-rabbit IgG and stained with BCIP/NBT (5-bromo 4-chloro 3-indolyl phosphate/ nitro blue tetrazolium).

Ubiquitination of ER *in vivo*: Rats were ovariectomized under light ether anaesthesia and were sacrificed 6, 24, 48 and 72 hours after ovariectomy. As controls, two rats were sacrificed immediately after ovariectomy. Ten days after ovariectomy, a group of rats were implanted subcutaneously with estradiol-bees wax pellets and sacrificed 6, 24, 48 and 72 hours after the implantation. Two rats were sacrificed immediately after implantation and were used as controls.

All the uteri were excised and the adhering fat tissue was removed. The uteri were homogenized in TEMN buffer and the cytosol was prepared. Cytosol (1mg protein) was immunoprecipitated either with anti-ER IgG (1:50) or with anti-ubiquitin IgG (1:50) as described before. The immunoprecipitates were subjected to SDS-PAGE in 7.5% gels, in duplicates. One of the gels was stained with silver nitrate. Proteins in the other gels were transferred to nitrocellulose membranes; the anti-ER immunoprecipitated samples were crossreacted with ubiquitin antibody while the anti-ubiquitin immunoprecipitated samples were cross reacted with anti-ER antibody.

Effect of estradiol on ubiquitin levels: Rats were ovariectomized under light ether anaesthesia and one group of rats was sacrificed ten days after ovariectomy. Another group of rats was implanted subcutaneously with estradiol-beeswax pellets and sacrificed 24 hours after

implantation. As controls two rats were sacrificed prior to ovariectomy. Uteri, liver, kidney and diaphragm were excised from the different groups of rats and ubiquitin was isolated from 250 mg tissue in each case following the procedure of Parakh and Kannan (11). The ubiquitin thus obtained was subjected to SDS-PAGE on 15% gels and stained with silver nitrate.

Miscellany: SDS-PAGE was performed as described by Laemmli (12) and the gels were stained with silver nitrate following the procedure of Blum *et al.* (13). Western blotting analysis was performed according to the procedure developed by Towbin *et al.* (14) and Blake *et al.* (15).

RESULTS

During ubiquitination, ubiquitin molecules are conjugated to a protein via an isopeptide bond between the C-terminal glycine of the ubiquitin and the ε-NH_2 of an internal lysine of the protein under consideration. This isopeptide bond is not destroyed by SDS or β-mercaptoethanol. Thus ubiquitinated proteins are seen in SDS gels as a ladder of higher molecular weight species. Using the above principle, we wanted to examine: 1-whether the estrogen receptor was ubiquitinated; and 2-whether estrogen receptor ubiquitination was influenced by estradiol.

The experimental protocol to identify ubiquitinated ERs was to immunoprecipitate them with either the ER IgG or the ubiquitin IgG. The higher molecular weight species in the anti-ER or the anti-ubiquitin immunoprecipitates were to be analyzed for their cross reactivity with ubiquitin and ER antibodies respectively.

UBIQUITINATION OF ER IS ENHANCED BY ESTRADIOL *IN VITRO.*

Experiments were planned to identify the ER-ubiquitin conjugates in rat uteri and also to study the influence of estradiol on the ER-ubiquitin conjugation *in vitro*. The experiments were carried out as described in 'materials and methods'.

The silver stained gels of the anti-ER precipitated samples showed protein bands of increasing molecular weights along with increase in the time of incubation with estradiol. Along with the appearance of the high molecular weight species, the concentration of the 66 kDa protein (ER) showed a decrease during the first two hours of incubation. In the next two hours, the high molecular weight species increased to a limited extent while the 66 kDa protein concentration returned to normal, suggesting *'de novo'* protein synthesis (Fig 1A). The blot that was exposed to ER antibody, showed a similar protein profile. The high molecular weight bands crossreacted with the ER antibody, suggesting the ubiquitination of estrogen receptor. The concentration of the ER (66 kDa protein) decreased during the first 120 minutes of incubation and returned to the normal level within the next two hours. A distinct increase in the higher molecular weight species was noticed following incubation with estradiol (Fig 1B). The blot that was exposed to the ubiquitin antibody also showed a similar pattern. A 66 kDa protein also crossreacted with the ubiquitin antibody. An experiment was performed to check whether the ER itself showed any crossreactivity with anti-ubiquitin. The ubiquitin antibody did not crossreact with the ER (data not shown). Thus this 66 kDa protein seen in the anti-ubiquitin stained blots could possibly be another protein that co-precipitated with the ubiquitinated ERs. The ubiquitin antibody crossreacted with the high molecular weight species confirming that these high molecular weight proteins are indeed ubiquitinated ERs (Fig 1C). The intensity of staining with ubiquitin antibody increased with increase in the molecular weight of the protein, as observed in the blots. This could easily be

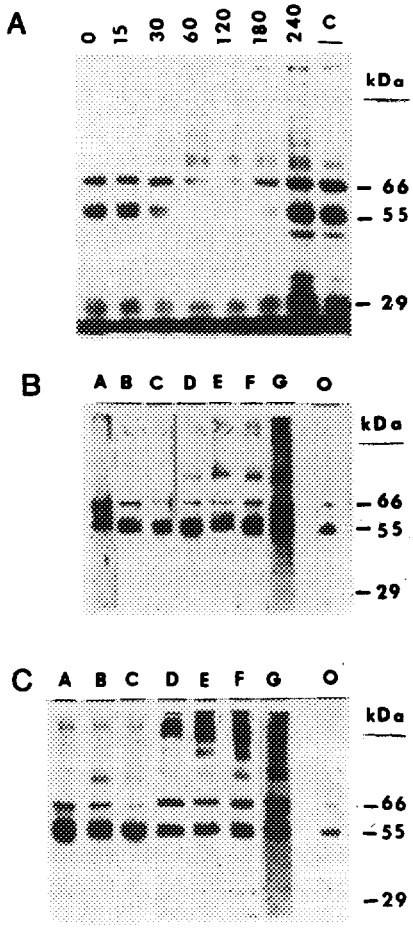

Figure 1. Ubiquitination of ER is enhanced by estradiol *in vitro*.
A. Uteri from ovariectomized rats were incubated in DMEM at 37°C in the presence of 20 nM estradiol for 0,15,30,60,120,180 and 240 minutes. One set of incubations was performed in the absence of estradiol for 240 minutes (C). Cytosol from each of these incubations was precipitated with anti-ER IgG (1:50). The immunoprecipitates were subjected to SDS-PAGE on 7.5% gels and stained with silver nitrate. The molecular weight markers (kDa) are indicated. The lane numbers indicate minutes of incubation with estradiol.
B & C. Uteri from ovariectomized rats were incubated in DMEM at 37°C in the presence of 20 nM estradiol for 0 (A), 15 (B), 30 (C), 60 (D), 120 (E), 180 (F), and 240 (G) minutes. One set of incubations was done in the absence of estradiol for 240 minutes (0). The cytosol (1 mg protein) of these uteri was immunoprecipitated in duplicates with anti-ER IgG. The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. One of the blots was incubated with anti-ER antibody (1:50) (**1B**) and the other with anti-ubiquitin antibody (1:50) (**1C**) overnight. The blots were washed and re-exposed to alkaline phosphatase coupled anti-rabbit IgG (1:1000). The blots were stained with BCIP/NBT.

explained. The higher molecular weight bands represent protein complexes with a single molecule of ER conjugated with increasing number of ubiquitin molecules. The greater the number of ubiquitin molecules, the greater the intensity of staining with the ubiquitin antibody.

To demonstrate whether the increase in ubiquitin-ER conjugation in the presence of estradiol was specific, uteri from ovariectomized rats were incubated in DMEM for 60-120

minutes in the presence of 20 nM progesterone, testosterone or dexamethasone. Rat uterine cytosol was immunoprecipitated with anti-ER IgG and crossreacted with the ubiquitin antiserum. No high molecular species were seen in the cytosol of the progesterone, dexamethasone and testosterone treated uteri. (data not shown).

UBIQUITINATION OF ER IS ENHANCED BY ESTRADIOL *IN VIVO*.

The previous experiment demonstrated that the ER was ubiquitinated *in vitro* and also that the ubiquitination was enhanced in the presence of estradiol. It was important to confirm this result *in vivo* and also to examine the influence of withdrawal of estradiol on the ubiquitination of the ER. The experiments were carried out as described in the materials and methods'.

The silver stained gels of the anti-ER (Fig 2A) and anti-ubiquitin (Fig 2B) precipitated samples showed high molecular weight species (ER-ubiquitination) upto 24 hours following ovariectomy. The content of high molecular weight species decreased 48 hours after ovariectomy (Fig 2A and B; left panel). These proteins crossreacted with both anti-ubiquitin (Fig 2A; right panel) and anti-ER antibodies (Fig 2B right panel).

The higher molecular weight species increased along with the advance in time during the first 24 hours following *in vivo* exposure to estradiol. Following this a decrease in the high molecular weight bands was noticed in both anti-ER precipitated (Fig 3A; left panel) and anti-ubiquitin precipitated (Fig 3B; left panel) gels. In both the gels, the high molecular weight species crossreacted with both anti-ubiquitin (Fig 3A; right panel) and anti-ER antibodies (Fig 3B; right panel).

High intensity of staining of the higher molecular weight bands by the ubiquitin antibody was also observed in these experiments. The highest molecular weight band represents a single molecule of ER conjugated with several molecules of ubiquitin. As one comes down the ladder, the number of ubiquitin molecules conjugated to the ER reduce which would be reflected in the reduced intensity of staining by anti-ubiquitin. The intensity of staining was directly proportional to the number of ubiquitin molecules conjugated to ER (Figs 2A and 3A right panel).

The effect of estradiol on ubiquitin levels *in vivo* was also studied as described in the 'Materials and methods'. Ovariectomy (UO) resulted in a distinct increase in the levels of ubiquitin in the rat uterus as compared to those in the control rat uterus (UC) (Fig 4). Implantation of estradiol resulted in a marked decrease in the levels of ubiquitin (UI). A similar effect of estradiol was seen on the ubiquitin levels in the rat liver (LO, LI of Fig 4). Estradiol did not show any marked effect on the levels of ubiquitin in the rat kidney (KO, KI) and diaphragm (DO, DI) (Fig 4).

DISCUSSION

Protein ladders were observed in the anti-ER and anti-ubiquitin immunoprecipitates of the rat uterine cytosol preparations under varying experimental conditions. These proteins crossreacted with both anti-ubiquitin and anti-ER antibodies suggesting that they are ER-ubiquitin conjugates. The ubiquitin-ER conjugation can also be referred to as the ubiquitination of ER. The ubiquitination of ER was enhanced by estradiol as observed in both *in vivo* and *in vitro* experiments. Withdrawal of estradiol *in vivo* decreased the ubiquitination of the ER. The increase

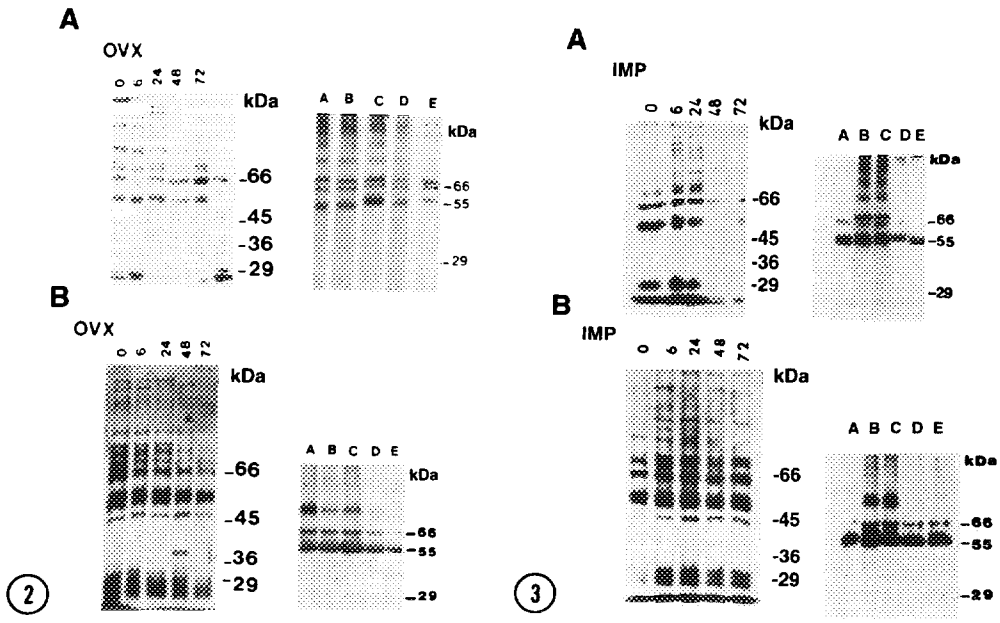

Figure 2. Ubiquitination of estrogen receptor decreases in rat uteri upon withdrawal of estradiol following ovariectomy.

Rats were ovariectomized and sacrificed 0, 6, 24, 48 and 72 hours after ovariectomy. One mg (protein) of uterine cytosol was immunoprecipitated with anti-ER IgG (1:50) (**2A**) or anti-ubiquitin IgG (1:50) (**2B**) as described in the methods. The immunoprecipitates were subjected to SDS-PAGE on 7.5% gels in duplicates. One set of gels was stained with silver nitrate (left panel). The lane numbers indicate hours after ovariectomy. The duplicate gels were transferred to nitrocellulose membranes. Blot **A** was exposed to anti-ubiquitin antibody (**2A;** right panel). Blot **B** was exposed to anti-ER antibody (**2B;** right panel). The blots were washed and re-exposed to alkaline phosphatase coupled anti-rabbit IgG (1:1000). The blots were stained with BCIP/NBT. The lane letters A, B, C, D, and E indicate 0, 6, 24, 48, and 72 hours respectively after ovariectomy.

Figure 3. Ubiquitination of ER is enhanced by estradiol *in vivo*.

Ovariectomized rats were implanted with estradiol-beeswax pellets. Rats were sacrificed 0, 6, 24, 48 and 72 hours after implantation. The uterine cytosol (1 mg protein) from all the experimental groups was immunoprecipitated with anti-ER IgG (1:50) (**A**) or anti-ubiquitin IgG (1:50) (**B**). The immunoprecipitates were subjected to SDS-PAGE on 7.5% gels in duplicates. The gels were stained with silver nitrate (left panel). The lane numbers indicate hours following hormone-wax implantation. The duplicate gels **A** and **B** were transferred to nitrocellulose membranes (right panel). Blot A was incubated with anti-ubiquitin antibody (1:50) and blot B was incubated with anti-ER antibody (1:50) overnight. The blots were re-exposed to alkaline phosphatase coupled anti-rabbit IgG (1:1000). The blots were stained with BCIP/NBT. A, B, C, D, and E indicate 0, 6, 24, 48 and 72 hours respectively after hormone-wax implantation.

in ubiquitination of ER in response to estradiol is specific as the other steroids, progesterone, testosterone, dexamethasone did not show any effect on the ubiquitination of ER (data not shown). Studies on the effect of estradiol on levels of ubiquitin in the different tissues of rat suggested that the effect of estradiol was specific to its target tissues, uterus and liver. The levels of ubiquitin in the rat uterus were inversely proportional to the levels of ubiquitination of the estrogen receptor. The decrease in the ubiquitination of ER, 10 days after ovariectomy, probably resulted in the increase in ubiquitin levels in the rat uterus. After implantation when increased ubiquitination of estrogen receptor was observed, the ubiquitin levels in the rat uterus showed a

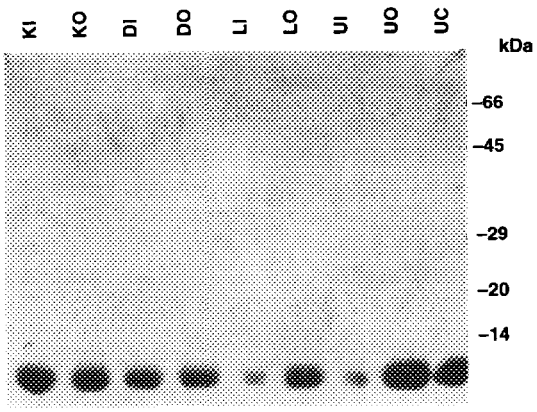

Fig.4. Effect of estradiol on ubiquitin levels.

Rats were ovariectomized and one group of rats was sacrificed ten days after ovariectomy. Another group of ovariectomized rats was implanted with estradiol-beeswax pellets and sacrificed 24 hours after implantation. As controls two rats were sacrificed prior to ovariectomy. Ubiquitin was isolated from the following tissues as described in the 'methods': Uteri, liver, kidney and diaphragm. The ubiquitin thus obtained was subjected to SDS-PAGE on 15% gels and stained with silver nitrate. U: Uterus; L: Liver; K: Kidney; D: Diaphragm. O and I represent ubiquitin isolated from the ovariectomised or estradiol-implanted rats, respectively. e.g., UO: ubiquitin from the uterus of ovariectomized rats; UI: ubiquitin from the uterus of estradiol-implanted rats. UC: ubiquitin isolated from the uterus of the control rats.

marked decrease suggesting that the ubiquitin molecules were probably used in the conjugation to estrogen receptor. Estradiol did not seem to influence the synthesis of ubiquitin. The changes in the ubiquitin levels following ovariectomy and implantation are probably due to the decreased or increased ubiquitination of estrogen receptor.

Based on the above observations, we wish to propose that the ER is degraded by the ubiquitin pathway as the ubiquitination of ER is enhanced by estradiol. The conjugation of ER to ubiquitin precedes degradation of the estrogen receptor. Thus these results show that estradiol enhances degradation of the ER. This agrees with the earlier findings of Sarff and Gorski (6) that ER levels in the cell decrease in the presence of estradiol. The immediate increase in ER ubiquitination observed during the first 24 hours following *in vivo* exposure of the rats to estradiol and the subsequent decrease in the reaction immediately thereafter is possibly indicative of the fact that the ubiquitination was dependent on a "threshold" level of circulating estradiol.

Based on what is currently known about the enzymes involved in ubiquitination it may be predicted that ER is conjugated to ubiquitin by the three ubiquitin conjugating enzymes, E1, E2 and E3 (5). The ubiquitin-ER conjugates are then recognized by the ubiquitin degradation enzymes, proteasomes, that degrade ER and release ubiquitin molecules for further use in the conjugation. This assumption needs to be confirmed by the isolation of E1, E2 and E3 enzymes from the rat uterus and the demonstration of ubiquitination of ER *in vitro* in the presence of these enzymes. The work presented here is a basic study to identify the pathway by which ER is degraded and is the first report ever regarding the ubiquitination of the ER or of any steroid receptor. Further work needs to be done to identify the different steps in the molecular pathway.

Phytochrome, cyclin, p53 and Mat α2 repressor have been identified to be degraded by the ubiquitin pathway following a similar experimental protocol as described here i.e.,

immunoprecipitation with the protein antibody and cross-reaction with anti-ubiquitin (16-19)

Acknowledgments: This work was supported by a research grant to RVT funded by the Department of Atomic Energy, Govt. of India (Sanction No.4/22/92G). PBN wishes to acknowledge CSIR for the Senior Research Fellowship.

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