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Cellular internalization and trafficking of 20 KDa human growth hormone

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ABSTRACT

Twenty kilodalton human growth hormone (20K-GH) is the second most abundant GH isoform after the twenty-two kilodalton human growth hormone (22 K-GH) isoform. 20K-GH exhibits similar but not identical physiological activities as that of 22K-GH. The cell behaviour of 22K-GH has been extensively studied, but little or no information has been reported regarding 20K-GH. Here, we focussed on the internalization of 20K-GH. We found that the internalization of 20K-GH is rapid and occurs in a time- and dose-dependent manner. 20K-GH internalization is mediated by GHR. It appears that the internalization of 20K-GH and GHR into the cytoplasm is mediated by clathrin and/or caveolin. The current study indicates that 20K-GH can internalize into the cytoplasm and suggests that the internalized 20K-GH may exhibit different functions from those of 22K-GH.

1. Introduction

Growth hormone (GH) exhibits important bioactivities in the regulation of growth and development (Brooks and Waters, 2010; Waters, 2016). GH exists in multiple forms of the GH proteoform in blood circulation, within which twenty kilodalton human growth hormone (20K-GH, consisting of 176 amino acids, representing 6% of all circulating plasma GH) is the most abundant hGH isoform except for twenty two kilodalton human growth hormone (22K-GH, consisting of 191 amino acid, representing 21% of all circulating GH) (Celniker et al., 1989; Leung et al., 2002; Popii and Baumann, 2004; Wada et al., 1998; Wu et al., 2010). 20K-GH is produced via alternative splicing of pituitary 22K-GH mRNA, resulting in the deletion of residues 32–46 of 22K-GH (Baumann, 1991).

Based on a literature review, it was found that 20K-GH displays similar but not absolutely identical biological activities with that of 22K-GH. It has been reported that 20K-GH exhibit equipotent activities with that of 22K-GH in cell-proliferation experiments (Roswall et al., 1996). Wada et al reported that 20K-GH and 22K-GH have the same binding affinities for hGHR and exhibit the same biological activities (Wada et al., 1998). On the other hand, Nuoffer et al (2000) found that at normal physiological concentrations, both 20K-GH and 22K-GH exert similar effects on GHR/GHBP gene transcription. However, under the condition of a supraphysiological concentration, 22K-GH and 20K-GH exert different biological activities. Takahashi et al (2001) reported that the diabetogenicity of 20K-GH and 22K-GH is different; 20K-GH is weaker in leading to insulin resistance compared with 22K-hGH, which suggests that 20K-GH may have important clinical benefits. In addition, Satozawa et al found that 20K- and 22K-GH exhibit different effects on water retention in rats (Satozawa et al., 2000).

Previous studies have mainly focused on comparing the physiological functions and roles of 22K-GH and 20K-GH. The cell behaviour of 22K-GH has been reported in detail. The internalization of 22K-GH was via the pathways of clathrin-mediated and caveolin-mediated endocytosis (Lobie et al., 1999; van Kerkhof et al., 2001). The internalized 22K-GH is differentially sorted into distinct populations of early endosomes (such as Rab4- and Rab5-positive endosome) (Lan et al., 2018). The cargo molecules (22K-GH) are then delivered to different organelles (such as lysosomes, mitochondria, Golgi apparatus or endoplasmic reticulum) (Lobie et al., 1994). In addition, 22K-GH also can transport into the nucleus, where it may play an important role (Lan et al., 2018). However, until now, only little has been known regarding the cell behaviour of 20K-GH.

Here, we studied the cell behaviour of 20K-GH and found that 20K-GH was internalized into the cytoplasm in a time-dependent manner, reaching different organelles. It has been reported that 22K-GH not only exerts its functions on the cell membrane but also in the cytoplasm and nuclei. Therefore, the current work implies that the bioactivities

Abbreviations: 22K-GH, 22 KDa growth hormone; 20K-GH, 20 KDa growth hormone; GHR, growth hormone receptor

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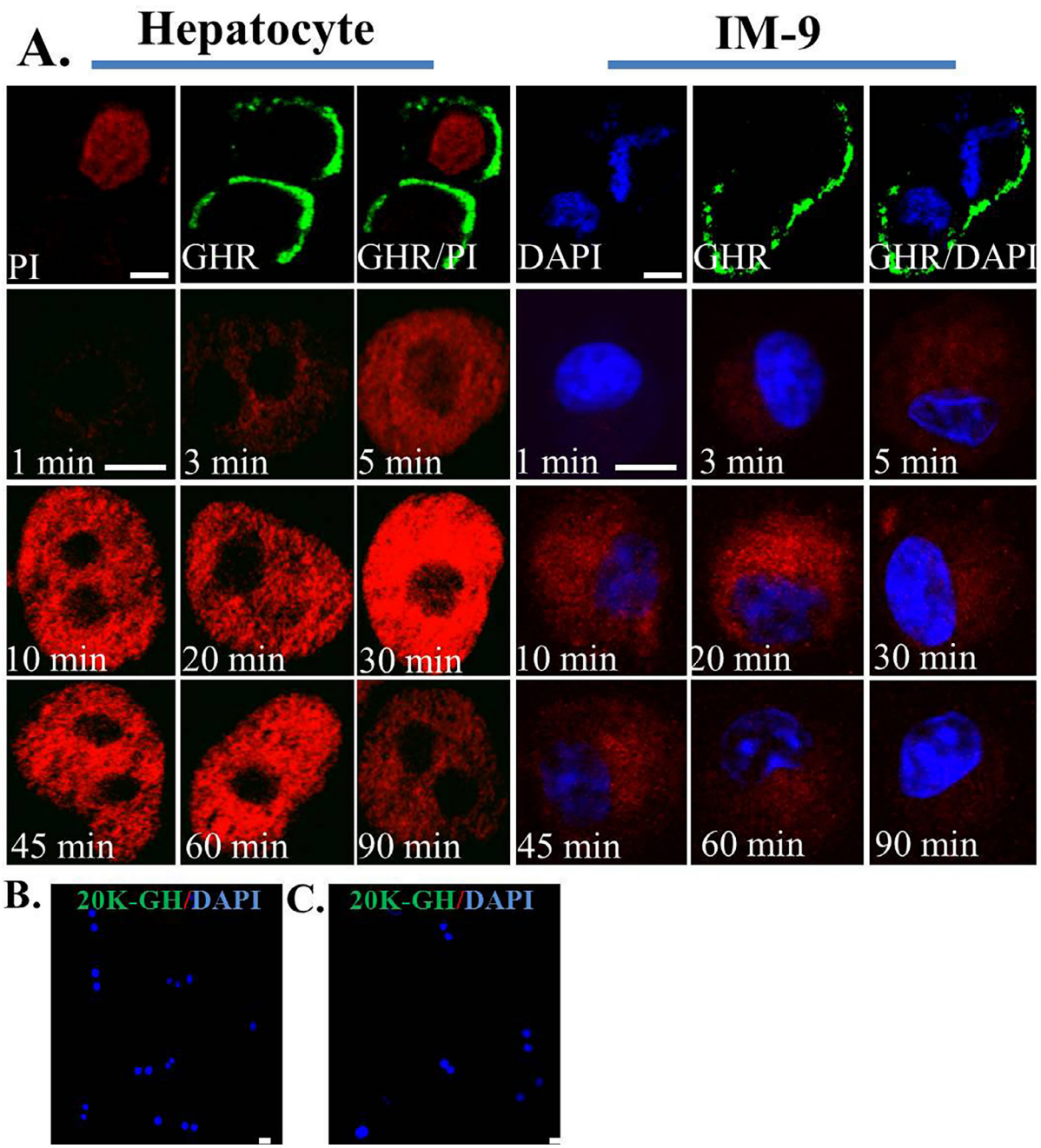

Fig. 1. Dynamics of 20K-GH's internalization. A. The rat hepatocytes and IM-9 cells were cultured in serum-free medium for 2 h. After washing, the cells were incubated with Alexa Fluor 555-20K-GH (20 nM) for the indicated times. After washing, the cells were fixed in 4% paraformaldehyde for 20 min. the cell nuclei were then strained with DAPI or PI. After three washes, the cells were visualized by confocal laser scanning microscopy (Olympus FV3000). Three images show GHR staining (green). B. The rat hepatocytes were incubated with Alexa Fluor 555-20K-GH and 200-fold excess of unlabeled 20K-GH. C. the hepatocytes were pre-incubated with anti-GHR antibody to block membrane-bound GHR. Bar = 10 μm.

exhibited by 20K-GH localized in cytoplasm should be explored. In addition, the current work also provides an explanation for why 20K-GH exhibits different bioactivities than those of 22K-GH.

2. Materials and methods

2.1. Reagent

Recombinant 20K-GH was obtained from Mybiosource. Recombinant 22 KDa human GH (hGH) was obtained from Sigma-Aldrich. Alexa Fluor 488 and Alexa Fluor 555 were purchased from Thermo Fisher Scientific. 20K-/22K-GH conjugated with Alexa Fluor 488 or Alexa Fluor 555 were prepared according to our previous described methods (Lan et al., 2014). The reagents for Western-Blotting were from Beyotime. Anti-GHR antibody was from Abcam. Functional activity of 20/22K-GH labeled with Alexa Fluor 488 and Alexa Fluor 555 were checked by Western blot analysis. The Nuclear/ Cytoplasmic Extraction Reagent Kits were obtained from pierce (USA).

2.2. Rat hepatocyte isolation

In this work, somatic cells, rat hepatocytes that endogenously and abundantly express GHR, were used as the cell model. 20K-GH can bind GHR and prolactin receptor (PRLR). To eliminate interference from

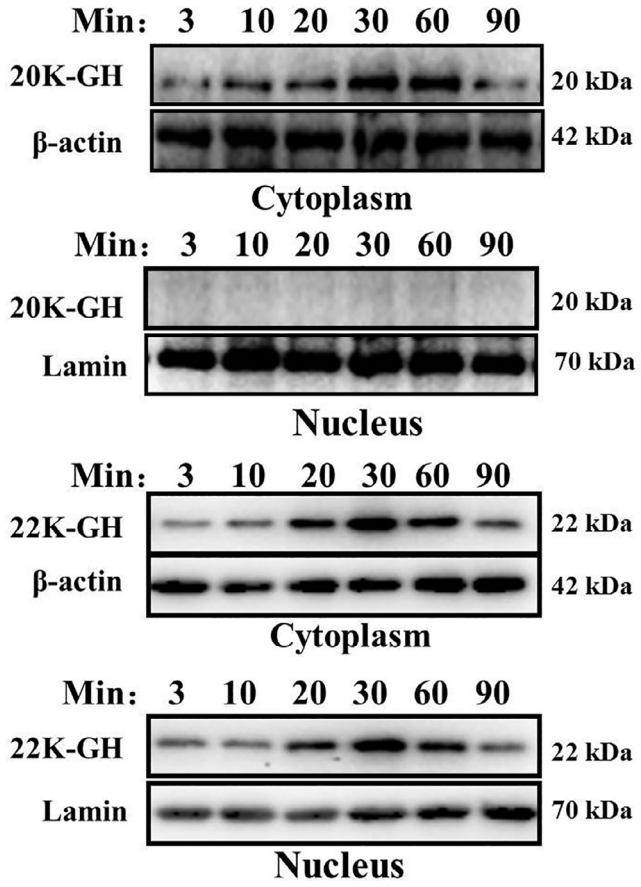

Fig. 2. Internalization of nuclear localization of 20K-GH/22K-GH by western-blot analysis. The rat hepatocytes were treated with 20K-GH for indicated time points. Whole, Nuclear and cytosol proteins of the cell samples were extracted by Nuclear and Cytosol Extraction Kit. The Nuclear and cytosol proteins were then subjected to Western-blot analysis. The figures shown are representative of three independent experiments.

PRLR, we isolated rat hepatocytes from PRLR gene knockout rats that were provided by Hua Cheng Medical and Biological Laboratories Co., Ltd. In the pre-treatment, we determined GHR expression and the signalling sensitivity of GH on hepatocytes from PRLR-gene-knockout rats and found that they were not significantly affected or changed. Rat hepatocytes were isolated according to our previous methods (Lan et al., 2015). The study was approved by the Animal Ethical Committee of Jilin Agricultural University.

In addition, IM-9 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were cultured as described previously (Silva et al., 1996).

2.3. Intracellular trafficking of 20K-GH

Freshly isolated hepatocytes and IM-9 cells were seed on confocal dish. The cells were cultured in serum-free medium for 2 h. After two washes with PBS, the cells were treated with 20K-GH conjugated with Alexa Fluor 555 (20 nM) for different times (1–90 min). After washing the cells, they were fixed in 4% paraformaldehyde for 20 min. After washing, the cells were checked by confocal laser scanning microscopy (CLSM, Olympus FV3000). At same time, the following control experiments were performed, which included: (a) competition with a 200-fold excess of unlabelled 20K-GH; (b) pre-incubation with a 5 mg excess of anti-GHR antibody.

2.4. Preparation of subcellular fractions

Rat hepatocytes were treated with 20K-GH under the same conditions as described above. After treatment, the rat hepatocytes were harvested at selected time points. The nuclear and cytosol fractions of the cell samples were extracted using the Nuclear and Cytosol Extraction Kit according to the manufacturer's instructions. The cytosol and nuclear extracts were then subjected to immunoprecipitation and western blot experiments using the indicated antibodies; the experimental details are described below.

2.5. Immunoprecipitation (IP)

Immunoprecipitation was performed by incubating nuclear/cytosol extraction with the indicated antibodies, after which, immunoglobulin-bound proteins were isolated using the IP Kit. The immunoprecipitated proteins were subsequently analyzed by western bolt.

2.6. Western-blot

Nuclear and cytosol proteins were separated by SDS–PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with the anti-GH overnight at 4 °C. After washing for three times with TBST, the membranes were incubated with HRP-labeled secondary antibody for 1 h at 37 °C. Following three washes with TBST buffer, the membranes were detected using the ECL detection system.

3. Results

3.1. 20K-GH internalization

Freshly isolated rat hepatocytes and IM-9 cells were used as a model to observe the internalization of 20K-GH. The cells were treated with 20K-GH at the indicated time points; the following experimental process was described in the Materials and Methods section. As indicated in Fig. 1A, at 1 min, little or no fluorescence signal was found in the hepatocytes and IM-9 cells; at 3–5 min, the cytoplasm showed some fluorescence signal, which indicated that 20K-GH was mainly distributed in the cytoplasm; at 10–60 min, 20K-GH was mainly localized in the cytoplasm, and the cytoplasm showed a strong fluorescence signal; and at 90 min, the cytoplasmic fluorescence signal showed a slight decline compared with that at 60 min. To confirm the fluorescence staining specificity of 20K-GH, several control experiments were also performed as described in the Materials and Methods. First, when rat hepatocytes were incubated with Alexa Fluor 555–20K-GH and a 200-fold excess of unlabelled 20K-GH, only a small fluorescence signal was detectable (Fig. 1B); second, the hepatocytes were pre-incubated with an anti-GHR antibody to block membrane-bound GHR, and little or no positive signal was detected when the cells were stimulated with Alexa Fluor 488–20K-GH (Fig. 1C). These control experiments confirm that the internalization of 20K-GH is specific.

In addition, western blotting experiments were also performed to confirm the cytoplasmic localization of 20K-GH. After 20K-GH treatment, the nuclear and cytosolic proteins of the cell samples were extracted by a Nuclear and Cytosol Extraction Kit. The nuclear and cytosol proteins were then analysed using western blotting. As shown in Fig. 2, the results also indicate that 20K-GH localized in the cytoplasm but not in the nuclei.

3.2. Co-localization of 20K-GH and 22K-GH in rat hepatocytes

The subcellular localization of 22K-GH was studied in detail; it was found that internalized 22K-GH is localized in a series of subcellular fractions, including lysosomes, mitochondria, and the Golgi apparatus. Here, to determine the subcellular localization of 20K-GH, we

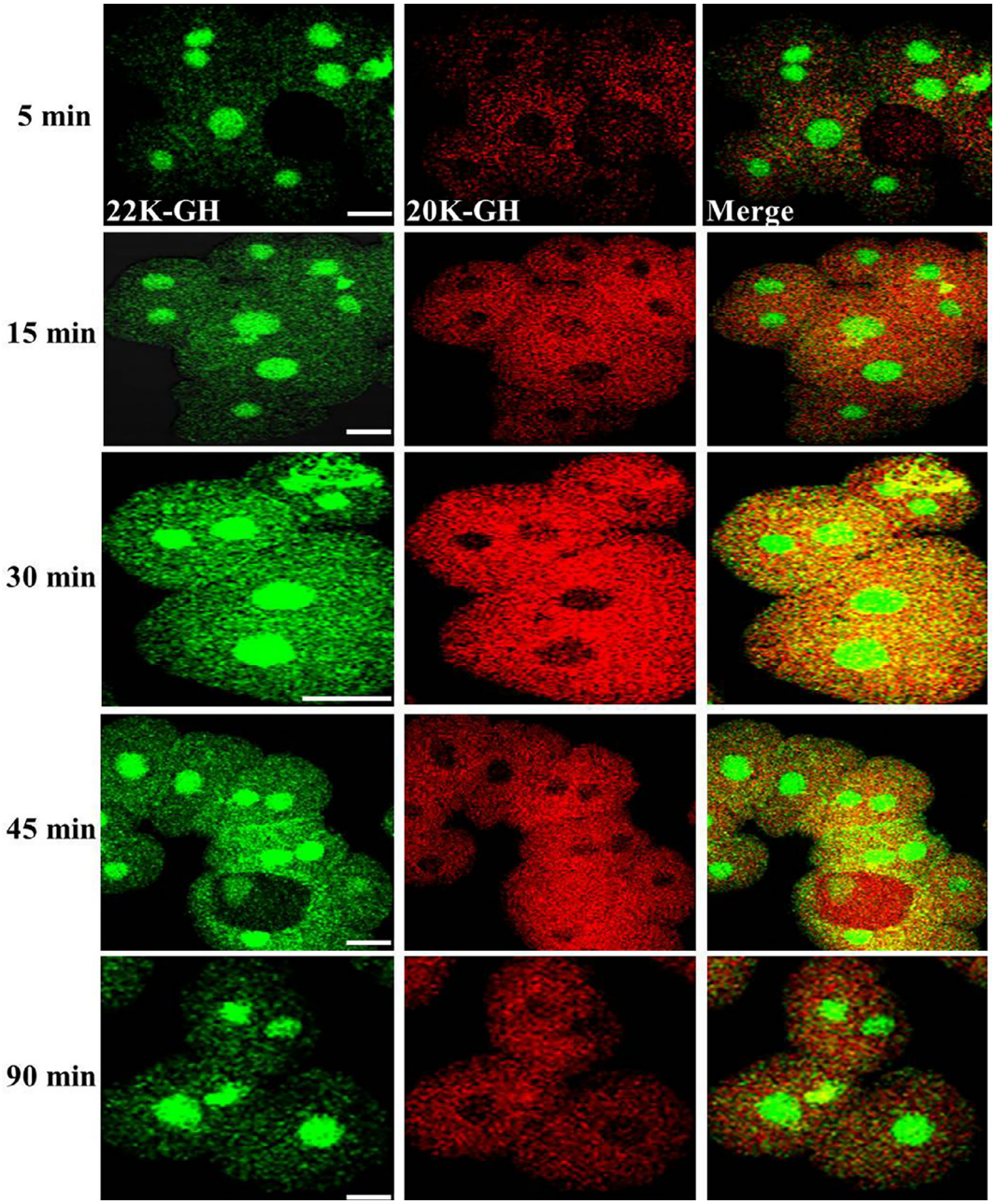

Fig. 3. Co-localisation of 20K-GH and 22K-GH. The rat hepatocytes were incubated with 20/22K-GH labeled with FITC or TRITC (red and green signal). After three washes, the cells were fixed in 4% paraformaldehyde for 20 min. the cell nuclei were then strained with DAPI. After three washes, the cells were visualized by confocal laser scanning microscopy (Olympus FV1000). Bar = 10 μm.

colocalized 20K-GH and 22K-GH (22K-GH was used as a reference substance). As shown in Fig. 3, in the cytoplasm, the pattern of subcellular localization of 20K-GH is similar to that of 22K-GH; these findings suggest that, similar to 22K-GH, 20K-GH is also localized in cellular organelles, such as lysosomes, mitochondria, and the Golgi apparatus. This remains to be further examined.

In addition, we found that 20K-GH could not translocate into the nuclei, different from 22K-GH. To further explore the mechanism for why 20K-GH cannot translocate into the nuclei, immunoprecipitation and western blot experiments were performed. We subjected cytoplasm extracts from rat hepatocytes that were treated with 20K-GH to immunoprecipitation with the indicated antibodies, followed by immunoblotting with the indicated antibodies. As indicated in Fig. 4, we did not detect the interactions between GHR and IMPα/β. However, the GHR/IMPα/β components were detected in the immunoprecipitates after 22K-GH treatment. These findings provide a possible explanation for why 20K-GH could not translocate into nuclei.

3.3. 20K-GH and GHR may exist in dimer form after internalization

To further analyse whether the internalized 20K-GH formed a dimer with GHR, cytoplasmic proteins were extracted after treatment with 20K-GH, followed by IP-WB and IFA analyses. As indicated in Fig. 5, we detected 20K-GH and GHR in the cytoplasm extract, which indicated that GH and GHR (at least partial), in a dimerization-dependent fashion (20K-GH-GHR complex), internalized into cells. The results also showed that the internalized 20K-GH/GHR was still active.

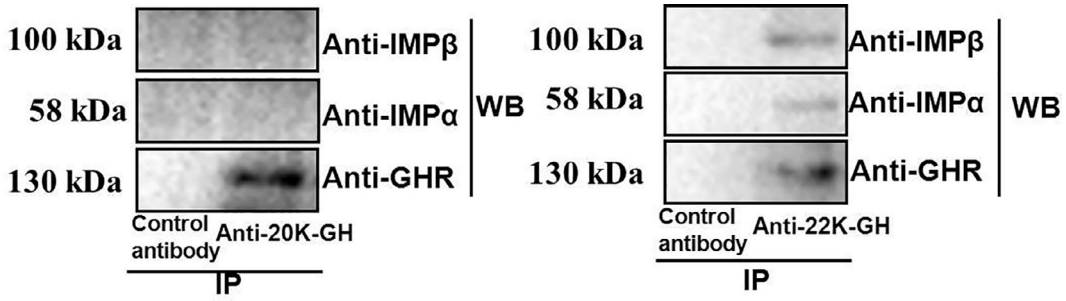

Fig. 4. Interactions between 20K-GH/GHR and IMPa/β complex. The rat hepatocytes were treated with 20K-GH for 30 min. Cytosolic and nuclear fractions of the cells were then isolated by nuclear/cytosolic extraction kit, after which, the cytosolic and nuclear proteins were subjected to immunoprecipitation with anti-GH antibody. Subsequently, Western blot analyses were performed with the indicated antibodies. The figures shown are representative of three independent experiments.

3.4. GHR is ubiquitinated under 20K-GH stimulation

Many studies have reported that ubiquitin is involved in 22KGH/GHR internalization. Therefore, we explored whether 20K-GH could induce GHR ubiquitination. As shown in Fig. 6, GHR was ubiquitinated under 20K-GH stimulation. To further detect whether the ubiquitin-proteasome pathway is involved in the 20K-GH-GHR internalization, MG132 was used to treat cells, and the results showed that 20K-GH-GHR internalization was partially inhibited, indicating that the ubiquitin-proteasome pathway is involved in the 20K-GH-GHR internalization, similar to that of 22K-GH.

3.5. Clathrin and caveolin may be involved in 20K-GH internalization

First, to further explore the mechanism of 20K-GH internalization into the cytoplasm, indirect immunofluorescence (IFA) was performed. It has been reported that clathrin and caveolin are involved in 22K-GH/GHR internalization. We explored whether 20K-GH could interact with clathrin and caveolin. As shown in Fig. 7, the colocalization signal for 20K-GH and clathrin was detected, suggesting that clathrin and caveolin may be involved in 20K-GH internalization.

3.6. The endocytic 20K-GH/GHR complex entered into the different types of endosomes

After 20K-GH/GHR endocytosis, 20K-GH/GHR targets different subcellular components. The endosome is required for 20K-GH/GHR cytoplasmic delivery. The colocalization signals for 20K-GH and EEA1 (an early endosome marker) were detected, indicating that 20K-GH/GHR entered into early endosomes. Endosomes can be further distinguished based on specific Rab GTPases. Endosomes that are enriched in different Rabs are strongly related to their destination. Rab5 is enriched in early endosomes, Rab4 and Rab 11 are enriched in recycling endosomes, and Rab7 and Rab9 are enriched in late endosomes, which can mediate trafficking to lysosomes for degradation. Colocalization experiments were performed to evaluate which endosomes 20K-GH/GHR entered, and the results indicated that 20K-GH/GHR entered Rab4 and Rab5-positive endosomes (Fig. 8), which explains why 20K-GH/

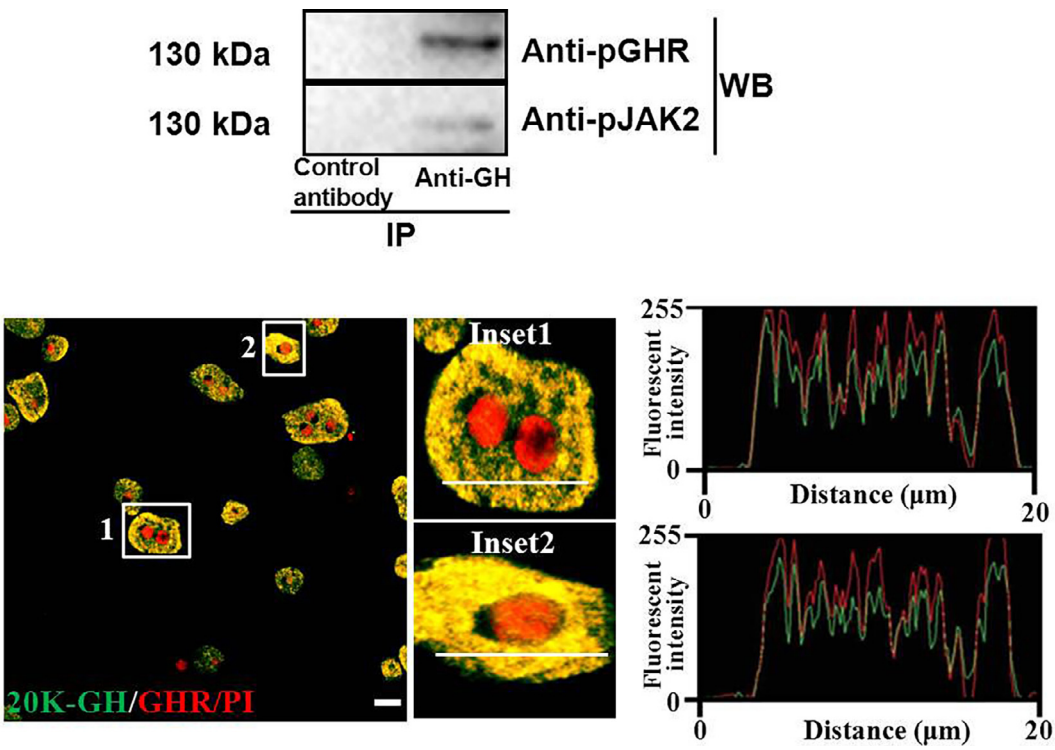

Fig. 5. Interactions within 20K-GH and GHR. The rat hepatocytes were treated with 20K-GH for 30 min. Cytosol and nuclear fractions of the cells were then isolated by Nuclear and cytosol extraction kit, after which, the cytosol and nuclear proteins were subjected to immunoprecipitation with anti-GH antibody. Subsequently, Western-blotting analyses were performed with the indicated antibodies. The figures shown are representative of three independent experiments.

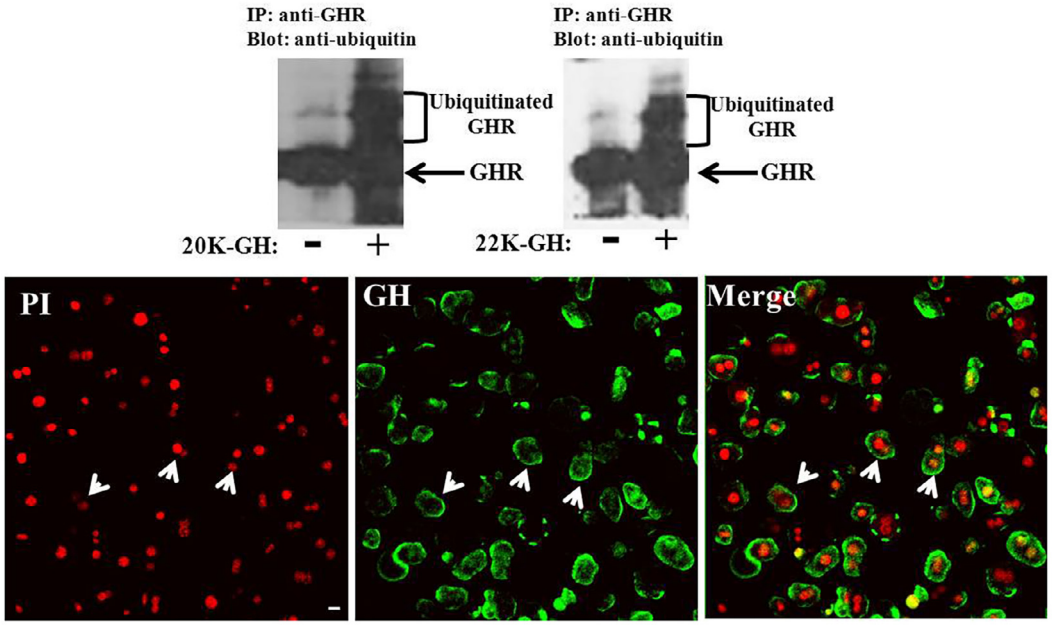

Fig. 6. A. GHR is ubiquitinated after 20K-GH treatment. The rat hepatyocytes were treated with 20K-GH for 30 min, after which, the samples was subjected to immunoprecipitation with anti-GH antibody. Subsequently, Western-blotting analyses were performed with the indicated antibodies. B. The 20K-GH internalization was inhibited by MG132 treatment. Bar = 10 μm.

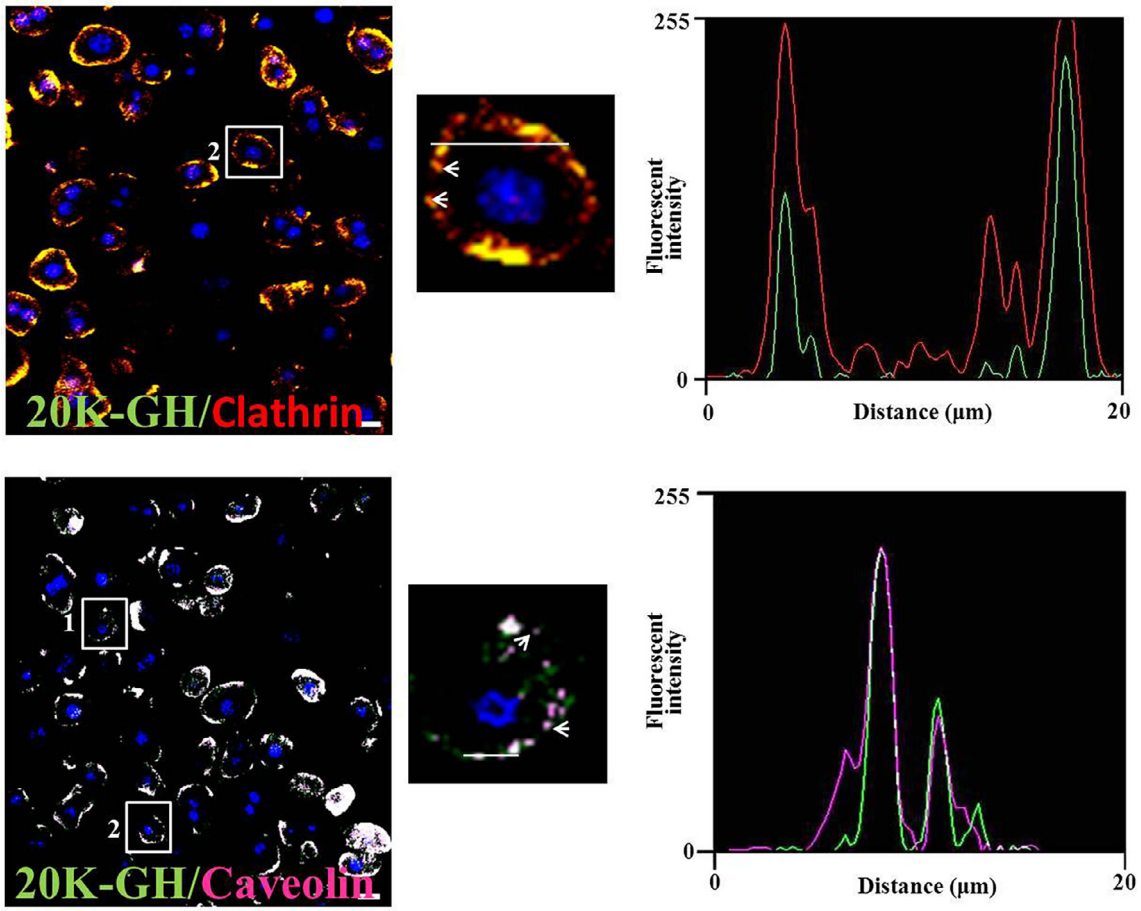

Fig. 7. 20K-GH/GHR's endocytosis. A. Colocalization of 20K-GH with clathrin or caveolin. The hepatocytes were treated with 20K-GH for 15 min, after which, the cells were fixed, blocked and permeabilized, and the cells were then incubated with the anti-caveolin antibody (abcam, ab2910) or anti-clathrin antibody (abcam, ab2731). After washing for three times, the cells were incubated with Alexa-conjugated secondary antibodies for 1 h in the dark. Optical sections showed colocalization signals. Bar = 10 μm. Arrowheads indicates colocalization signal.

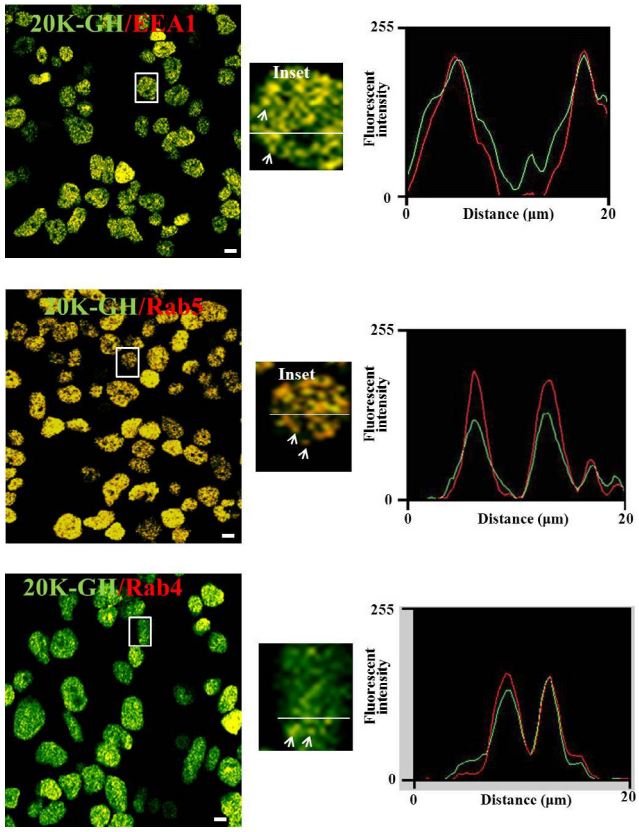

Fig. 8. Colocalization analyses with an indirect immunofluorescent assay (IFA). The hepatocytes were incubated with 20K-GH for 30 min, after which, the cells were fixed, blocked and permeabilized. The cells were then incubated with the indicated primary antibodies. After three washes with PBST, the cells were incubated with secondary antibodies. After three washes, the cells were visualized by confocal laser scanning microscopy (Olympus FV3000). Bar = 10 μm. Arrowheads indicates colocalization signal.

GHR showed different subcellular localizations.

4. Discussion

It has been extensively reported that 20K-GH exhibits similar but not identical functions and roles as those of 22K-GH. However, until now, there has been little information regarding the cell behaviour of 20K-GH. In the current study, we explored the cell behaviour of 20K-GH in rat cell models; we found that 20K-GH can internalize into the cytoplasm in a clathrin-mediated manner and then be targeted to different organelles. To our knowledge, this is first report regarding the cell behaviour of 20K-GH.

Accumulating evidence has shown that the nuclear localization of GHR induced by 22K-GH has important physiological functions (Conway-Campbell et al., 2008; Conway-Campbell et al., 2007). However, to date, the mechanisms via the nuclear-localized GH exerts its physiological roles remains to be revealed, and it appears that GH may form a dimer with GHR (GH-GHR) in the nuclei (Lobie et al., 1994; Mertani et al., 2003). In addition, it has been well-demonstrated that GHR nuclear localization is GH-dependent (Lan et al., 2017); previous studies have indicated that 22K-GH may form a dimer with GHR in the cell nuclei, but how the GH-GHR dimer exerts its functions remains to be understood. However, in the current study, we found that 20K-GH cannot translocate into cell nuclei. To further explore this question, we performed experiments and found that 20K-GH could not induce the interaction between GHR and IMPα/β, which provided an explanation for why 20K-GH could not induce GHR nuclear translocation. In addition, this finding also suggests that 20K-GH may exhibit different functions from that of 22K-GH.

The subcellular localization of 22K-GH has been extensively studied. 22K-GH was found to be localized in lysosomes, mitochondria, and the Golgi apparatus (Lan et al., 2018; Lobie et al., 1994). In the current study, we performed colocalization experiments; it was found that 20K-GH showed similar cytoplasmic localization with that of 22K-GH (Fig. 3). Previous studies have indicated that cytoplasm-localized GH-GHR still has the ability to transmit signals. In addition, many studies have shown that cytokines (e.g., EGF) can exhibit physiological functions based on their subcellular organelle localization. Therefore, the functions of organelle-localized 20/22K-GH deserve further study in the future.

In general, the internalization of 20K-GH includes two main steps: (1) 20K-GH endocytosis into the cytoplasm from the cell membrane; (2) cytoplasmic 20K-GH transportation into different organelles. The process of GH internalization from the cell membrane appears to involve the clathrin-coated pathway (van Kerkhof et al., 2001), caveolae (Lobie et al., 1999), and ubiquitin system (Strous et al., 1996). In addition, 20K-GH targeting different organelles may be involved in complex-molecular processing, possibly including multiple pathways. Although both 22K-GH and GHR have no typical nuclear localization signal (NLS), the interaction between 22K-GH-GHR and importin α/β in the cytoplasm was detected. In addition, Waters et al have reported that GHBP (namely, ECD of GHR) interacts with importin α/β (Conway-Campbell et al., 2007). However, until now, the exact molecular mechanism via which GH and/or GHR are translocated into the cell nuclei remains to be fully understood. Further studies need to be performed in the future. In the present work, we found that 20K-GH did not translocate into cell nuclei. We found that 20kGH-GHR could not interact with importin α/β, which may be a reason why 20K-GH/GHR fails to transport into the cell nuclei (Fig. 4). Except for nuclear localization, it has been reported that internalized GH still exhibits the ability to transmit signals in the cytoplasm. Therefore, the physiological functions of 20K-GH localized in the cytoplasm cannot be ignored.

In summary, in this work, we explored the cell behaviour of 20K-GH in rat hepatocyte models and found that 20K-GH is internalized into the cytoplasm in a time-dependent manner, which implies that further studies need to be conducted to explore the physiological functions and roles of the internalized 20K-GH. The current work lays the foundation for further exploration of the 20K-GH bioactive.

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Declaration of interest

The authors declare no conflict of interest.

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