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The expression of nerve growth factor in healthy and inflamed equine chondrocytes analysed by capillary western immunoassay

Susanne Nyström [a], Anna Kendall [b,*], Saritha Adepu [b], Anders Lindahl [a,c], Eva Skiöldebrand [b]

[a] Department of Clinical Chemistry, Sahlgrenska University Hospital, Bruna Stråket 16, 413 45 Gothenburg, Sweden
[b] Department of Pathology, Institute of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Box 7028, 750 07 Uppsala, Sweden
[c] Department of Laboratory Medicine, Institute of Biomedicine, Sahlgrenska Academy at the University of Gothenburg, 405 30 Gothenburg, Sweden

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ABSTRACT

Nerve Growth Factor (NGF) is a signalling molecule for pain and inflammation. NGF is increased in synovial fluid from osteoarthritic humans and animals, compared to healthy controls. Monoclonal antibody therapy directed against NGF has been approved to treat pain in osteoarthritic dogs but despite many years of trialling, therapy has not been approved for human use. One reason for this is that adverse reactions with rapidly progressing osteoarthritis has occurred in some individuals. More detailed knowledge of NGF expression in joints is needed. In this study, capillary-based Simple Western was used to analyse NGF in cultured equine chondrocytes. Chondrocytes were collected post mortem from three macroscopically healthy intercarpal joints and three intercarpal joints with mild osteoarthritic changes. The chondrocytes were expanded to passage one and seeded in chondrogenic medium to maintain the phenotype. On day four, cells were either stimulated with LPS or kept untreated in medium. All cells were harvested on day five. Wes analysis of lysates did not show mature NGF but two proforms, 40 and 45 kDa, were identified. Results were confirmed with western blot. The same proforms were expressed in chondrocytes from healthy and osteoarthritic joints. Acute inflammation induced by LPS stimulation did not change the forms of expressed NGF. Capillary Simple Western offers a sensitive and sample-sparing alternative to traditional western blot. However, confirmation of peaks is imperative in order to avoid misinterpretation of findings. In addition, in this case the method did not offer the possibility of quantification advertised by the manufacturers.

1. Introduction

Nerve Growth Factor (NGF) has since its discovery (Levi-Montalcini and Hamburger, 1951) been implicated to play a part in many inflammatory, neurodegenerative and painful signalling processes (Denk et al., 2017; Minnone et al., 2017a). Synovial fluid from osteoarthritic human, canine and equine joints have higher concentrations of NGF compared to synovial fluid from healthy joints (Isola et al., 2011; Kendall et al., 2021; Montagnoli et al., 2017) and NGF has been found to increase in human osteoarthritic chondrocytes (Iannone et al., 2002). NGF is produced by human and murine chondrocytes, synovial fibroblasts and macrophages (Ohashi et al., 2021; Pecchi et al., 2014; Takano et al., 2016; Takano et al., 2017) and immune cells such as human monocytes and mast cells express NGF receptors (Ehrhard et al., 1993; Nilsson et al., 1997). Morevover, NGF can induce angiogenesis (Nico et al., 2008) and was found to stimulate intra-articular nerve sprouting in a murine arthritis model (Ghilardi et al., 2012). These findings indicate that NGF plays a role in osteoarthritis-associated pain and inflammation. In 2021, monoclonal NGF-antibody therapies were launched to treat pain associated with osteoarthritis (OA) in dogs and cats. In humans, NGF-antibody therapy for OA related pain has been trialled for many years (Berenbaum et al., 2020) but has to date (2022) not received FDA approval. Adverse events in a small percentage of the treated patients with rapidly progressing OA caused a temporary halt in trials in 2010 (Hochberg, 2015). Trials have since been continued but he reason for these adverse reactions is still unknown.

NGF is secreted both in a mature form, mNGF (molecular weight of 13 kDa) and as a larger precursor, proNGF (Fahnestock et al., 2004).

* Corresponding author.
E-mail addresses: susanne.nystrom@clinchem.gu.se (S. Nyström), anna.kendall@slu.se (A. Kendall), saritha.adepu@slu.se (S. Adepu), anders.lindahl@clinchem.gu.se (A. Lindahl), eva.skioldebrand@slu.se (E. Skiöldebrand).

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Several different proNGF forms have been described in laboratory animals, with molecular weights between 17 to >100 kDa reported (Lee et al., 2001; Reinshagen et al., 2000; Soligo et al., 2015). The significance of the differences in specific proforms is not known. Initially, the precursor part was thought to be biologically inactive and important only to ensure proper folding and processing into mNGF (Edwards et al., 1988). This has since been disproven, and proNGF and mNGF have been shown to have varying functions due to different receptor binding properties (Ioannou and Fahnestock, 2017; Lee et al., 2001). Mature NGF is primarily neurotrophic whereas proNGF can be either neurotrophic if it interacts with the TrkA receptor, or apoptotic if it binds to the $p75^{NTR}$ receptor in combination with the co-receptor sortillin (Ioannou and Fahnestock, 2017). Altered mNGF/proNGF balance with increases in proNGF have been found in neurodegenerative diseases such as Alzheimer (Peng et al., 2004) and diabetic retinopathy (Mysona et al., 2015), as well as in juvenile inflammatory- and rheumatoid arthritis (Minnone et al., 2017b).

Commercial NGF-ELISA kits and antibodies are often directed at mNGF and can therefore detect a mixture of mNGF and proNGF in biological samples. (Malerba et al., 2016; Soligo et al., 2015). This has implications for research in OA, where most published studies have focused on determining NGF without differentiating between mature- and proforms (Aloe et al., 1992; Montagnoli et al., 2017; Stoppiello et al., 2014). It is currently unknown what role the mNGF/proNGF balance plays in the different OA disease stages.

The ProteinSimple® capillary western immunoassay method may be appropriate for further research into the expression of different forms of NGF. The method is based on capillary protein separation by molecular size. In a fully automated system, samples are loaded onto capillaries, separated and incubated with primary and secondary (HRP-conjugated) antibodies. Chemiluminescence is produced after addition of luminol-peroxidase and is detected at multiple exposure times. Only small amounts of protein are needed for detection. As analytes are automatically quantified by calculation of the area under the curve for the detected peaks, the system provides the opportunity for both quantitative and qualitative analysis.

The aim of this study was to investigate which forms of NGF are expressed in healthy and osteoarthritic equine chondrocytes. The aim was also to determine how acute inflammation induced by lipopolysaccharide (LPS) influences NGF expression. The hypothesis was that not only mNGF, but also proNGF would be expressed and that the expression would differ in healthy, acutely inflamed and chronically inflamed chondrocytes.

2. Materials and methods

2.1. Chondrocyte isolation and culture

The chondrocytes were obtained for previous projects (Ley et al., 2011; Skiöldebrand et al., 2019) and horses were euthanized for reasons unrelated to the study.

Articular cartilage samples were collected from six age-matched horses within 48 h post mortem; three horses had mild macroscopic osteoarthritic cartilage changes of the dorsal radial facet and three were without visible lesions. (For demographic data, see Supplementary Table 1.) Following aseptic preparation, the joint was incised and the articular cartilage was inspected macroscopically. The macroscopic classification of the joints was performed by a board certified veterinary pathologist (ECVP). Cartilage on the dorsal aspect of the radial facet of the third carpal bone was incised with a scalpel down to the bone and full-thickness cartilage samples were collected.

Samples were placed in sterile saline (0.9% NaCl) solution with gentamicin sulfate (50 mg/l) and amphotericin B (250 μg/ml). The cartilage samples were transported chilled (approx. 5 °C) to the laboratory. Isolation and expansion of chondrocytes were performed as previously described (Ley et al., 2011). Briefly, the chondrocytes were expanded to passage 1 and then seeded at 20,000 cells/cm^2 in chondrogenic medium to maintain the phenotype. On day 4, cells were stimulated with LPS (10 ng/ml, *Escherichia coli* 055:B5; List Biological Laboratories, Campbell, CA, USA) or kept untreated (controls) for 24 h. Cells were grown to confluence and harvested on day 5 and immediately frozen and stored at −80 °C until further analyses.

2.2. Protein determination

A protein determination assay was performed in accordance with the manufacturer's instructions using a detergent-compatible protein assay (Bio-Rad, Hercules, CA, USA) based on Lowry's method. The standard (0–4 mg/ml BSA) and samples were mixed with the reagents and incubated for 15 min at room temperature. The absorbance was read at 750 nm with a VersaMax microplate reader and analysed using SoftMax Pro 4.8 (Molecular Devices, Sunnyvale, CA, USA).

2.3. Wes

Capillary western analyses were performed on the ProteinSimple Wes system (ProteinSimple, San Jose, CA, USA), according to the manufacturer's instructions using a 12–230 kDa Separation Module (SM-W004) and either the Anti-Rabbit Detection Module (DM-001) or the anti-rat secondary antibody (HAF-005, R&D systems, Minneapolis, MN, USA) depending on the primary antibody used.

Lysates were thawed and diluted in 0.1× Sample Buffer (ProteinSimple) to an appropriate concentration and mixed with 5× Fluorescent Master Mix (containing 5× sample buffer, 5× fluorescent standard, and 200 mM DTT) and heated at 95 °C for 5 min. Four parts of diluted sample were mixed with one part Master Mix.

The denatured samples, biotinylated ladder, antibody diluent, primary antibody, HRP-conjugated secondary antibody, chemiluminescent substrate, and wash buffer were pipetted into the assay plate according to the manufacturer's instructions, and loaded on Wes. The separation electrophoresis and immunodetection steps were then fully automated.

Instrument default settings were adjusted to maximize the protein sensitivity and protein signal: stacking gel loading time 21 s, sample loading time 12.6 s, separation at 475 V for 30–35 min, blocking reagent 5 min, primary antibody 60 min, secondary antibody 30 min, luminol-peroxide chemiluminescence detection for ~15 min (exposures of 1-2-4-8-16-32-64-128-512 s).

The resulting electropherograms were inspected and automatic peak fits/detections were manually corrected when required. In order for a peak to be considered, a signal to noise (S/N) ratio >10 was required (Beekman et al., 2018). A shift in apparent molecular weights of ±10% was accepted as representation of the same peak. The intra-run CV for area under the curve was set to ≤15% according to information from the manufacturer (data not shown).

Data analysis was performed with the Compass Software (ProteinSimple).

Results in Compass are in the form of electropherograms, where the software reports data as graphs showing chemiluminescence versus apparent molecular weight (MW). Apparent MW is determined by using the peak signals from the protein standard ladder. The fluorescent standards present in the 5× Master Mix are used to adjust for any differences in migration within the capillaries. The software calculates peak areas using the Gaussian method and these peak areas were used to compare samples.

2.3.1. Primary antibodies

Two NGF antibodies were selected: ab6199 (rabbit polyclonal, Abcam, Cambridge, UK) and MAB2562 (rat monoclonal, R&D Systems). For ab6199, the immunogen was mouse native salivary gland β-NGF hence the antibody could potentially recognise both mature- and proforms of NGF. The MAB2562 immunogen was *E. coli*-derived recombinant human proNGF, AA19–241, hence the antibody is specific for

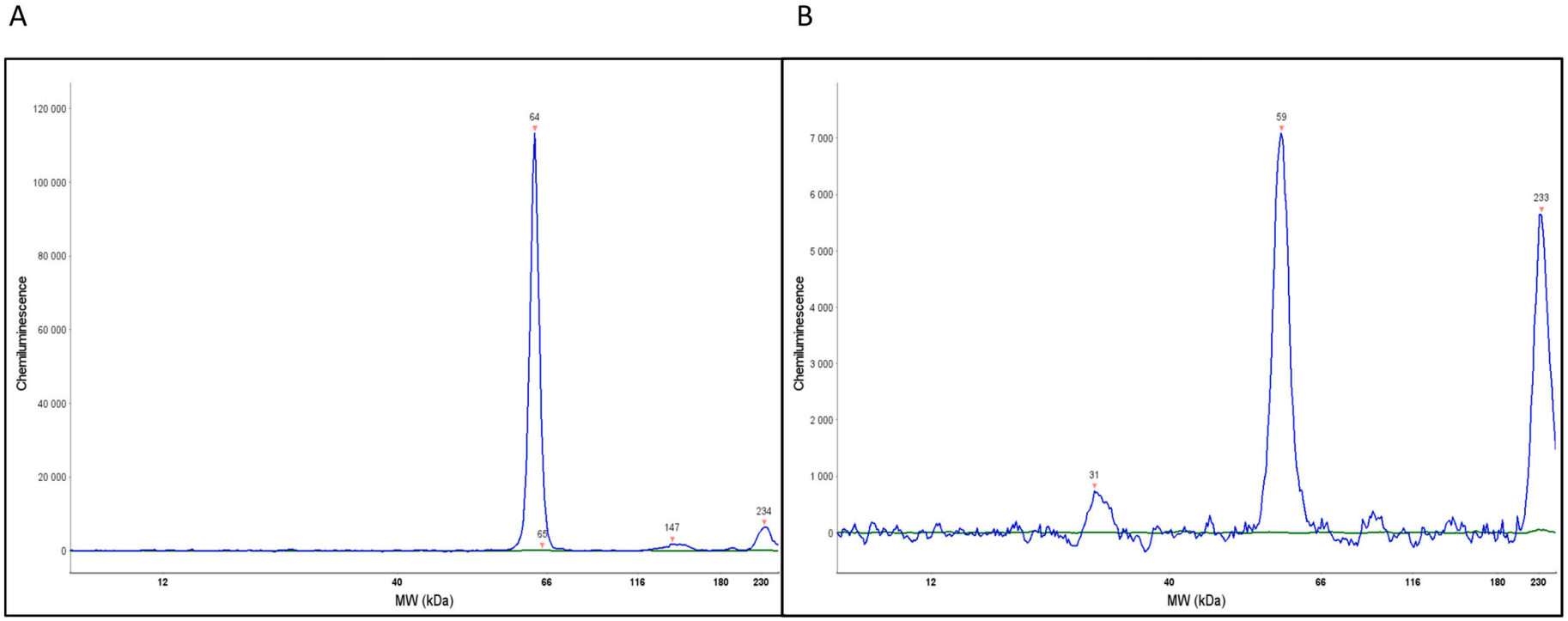

Fig. 1. A. Peaks detected by anti-NGF antibodies ab6199 (blue) and MAB2562 (green) in a solution of 0,015 mg/ml human serum albumin. There is unspecific binding to albumin with ab6199 but not with MAB2562. B. Peaks detected with the same antibodies in a solution of 0,007 mg/ml equine IgG protein. There is unspecific binding to IgG heavy and light chain with ab6199 but not with MAB2562. Peaks detected above 230 kDa are non-migrated proteins and are not considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proNGF only. Sequence identity between full-length human and full-length equine NGF is 94% (Uniprot BLAST). As an additional control the polyclonal antibody was compared to a no longer commercially available NGF antibody (H20, Santa Cruz Biotechnology, Heidelberg, Germany) that has previously been used in published research (Amagai et al., 2016; Bruno and Cuello, 2006; Malerba et al., 2016; Soligo et al., 2015). The two antibodies ab6199 and H20 were found to result in peaks at comparable molecular weights in the Wes system (data not shown).

2.3.2. Linearity and saturation

To ensure optimal sample protein load, a series of sample dilutions were made with a constant antibody concentration, to determine the linear range of the assay. Sample concentration for further analysis was performed within the linear range.

To determine optimal antibody concentration, our preferred sample concentration was tested against different antibody dilutions to determine the saturation point for the antibody. If saturation could not be reached, optimal antibody concentration was based on electropherogram characteristics such as baseline, S/N ratio and baseline to height ratio.

For detailed information on linearity and saturation see Supplementary table 2.

2.3.3. Controls

As many different molecular weights have been described for NGF, the NGF antibodies were run with recombinant NGF protein (256GF/CF, R&D Systems), protein load 0.00625–0.1 mg/ml, in order to better determine the specific NGF peaks.

A no lysate control (no protein control) was performed to check for primary antibody cross reactions. A no primary antibody control was also performed to check for cross reaction with the secondary antibody. In addition, all antibodies were tested against pure equine IgG (Bio-Rad), protein load 0.003–0.015 mg/ml and human serum albumin (Europa Bioproducts Ltd., Ipswich, UK), protein load 0.015 mg/ml, in order to identify any unspecific peaks.

2.4. Western blot

Analysis was carried out according to standard protocols. Protein concentrations were determined as described above. Western blot samples were prepared with 4× Laemmli sample buffer (Bio-Rad) with 10% β-mercaptoethanol, and sample in PBS. Three parts of diluted sample was mixed with one part sample buffer.

The solution was boiled at 95 °C for 5 min and samples were centrifuged for 1 min at 16,000 *g*. Nine microgram sample protein was loaded in each well of a pre-cast Mini-protean TGX stain-free gel (Bio-Rad) and run at 300 V for 15 min. The gel was activated and the blot was transferred to a low fluorescence PVDF membrane (Bio-Rad). After transfer the membrane was imaged for later protein normalisation. After blocking for 30 min in EveryBlot buffer (Bio-Rad), the membrane was incubated with primary antibody (MAB2562, 1:1000 dilution) at 4 °C overnight. Thereafter, the membrane was washed four times and incubated with secondary antibody (HAF-005, 1:1000) at room temperature for one hour. The membrane was once again washed four times and immediately imaged with the Chemidoc Touch Imaging System (Bio-Rad). Protein normalisation was performed by the Image Lab software (Bio-Rad) according to the manufacturer's instructions.

3. Results

3.1. Wes linearity and saturation

Linearity was obtained for both antibodies, however, saturation could not be obtained despite multiple experiments with increasing antibody concentrations up to 1:5 dilution, and decreasing protein concentrations until the disappearance of the specific peaks. Due to this,

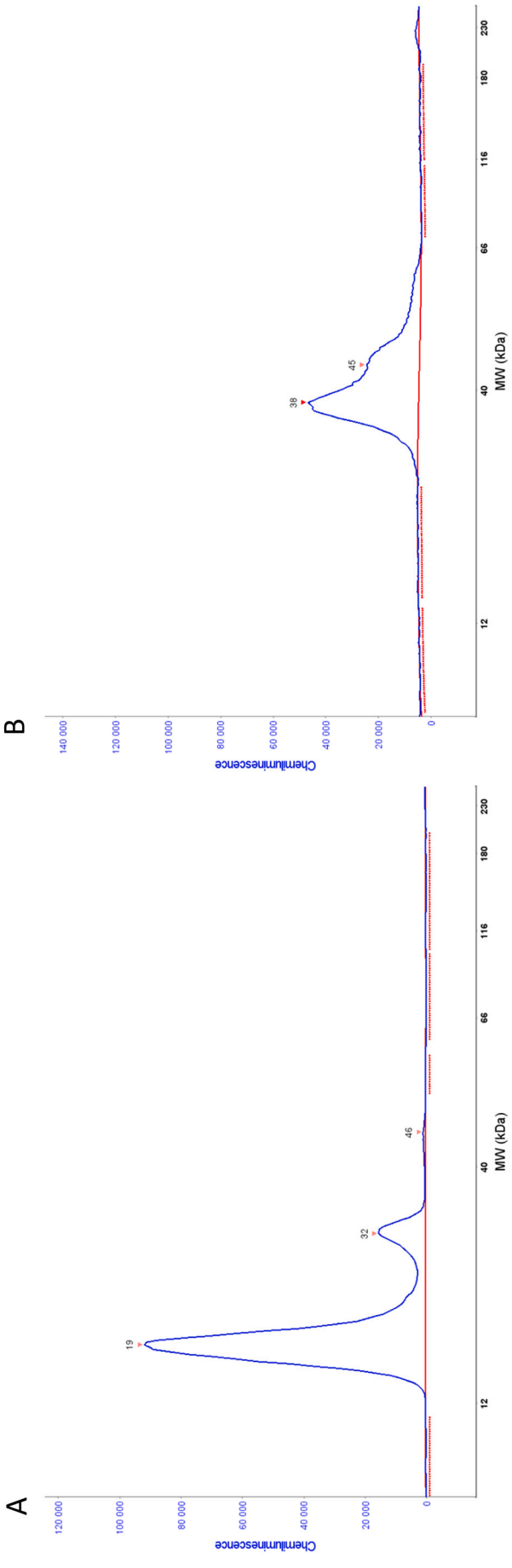

Fig. 2. Wes results for recombinant Nerve Growth Factor.
A. At 0.01 mg/ml protein load, the primary antibody ab6199 detects NGF at molecular weights 19, 32 and 46 kDa. The 19 kDa peak is interpreted as mNGF.
B. Protein load is increased to 0.1 mg/ml and primary antibody MAB2562, specific for proNGF, detects peaks at 38 and 45 kDa.

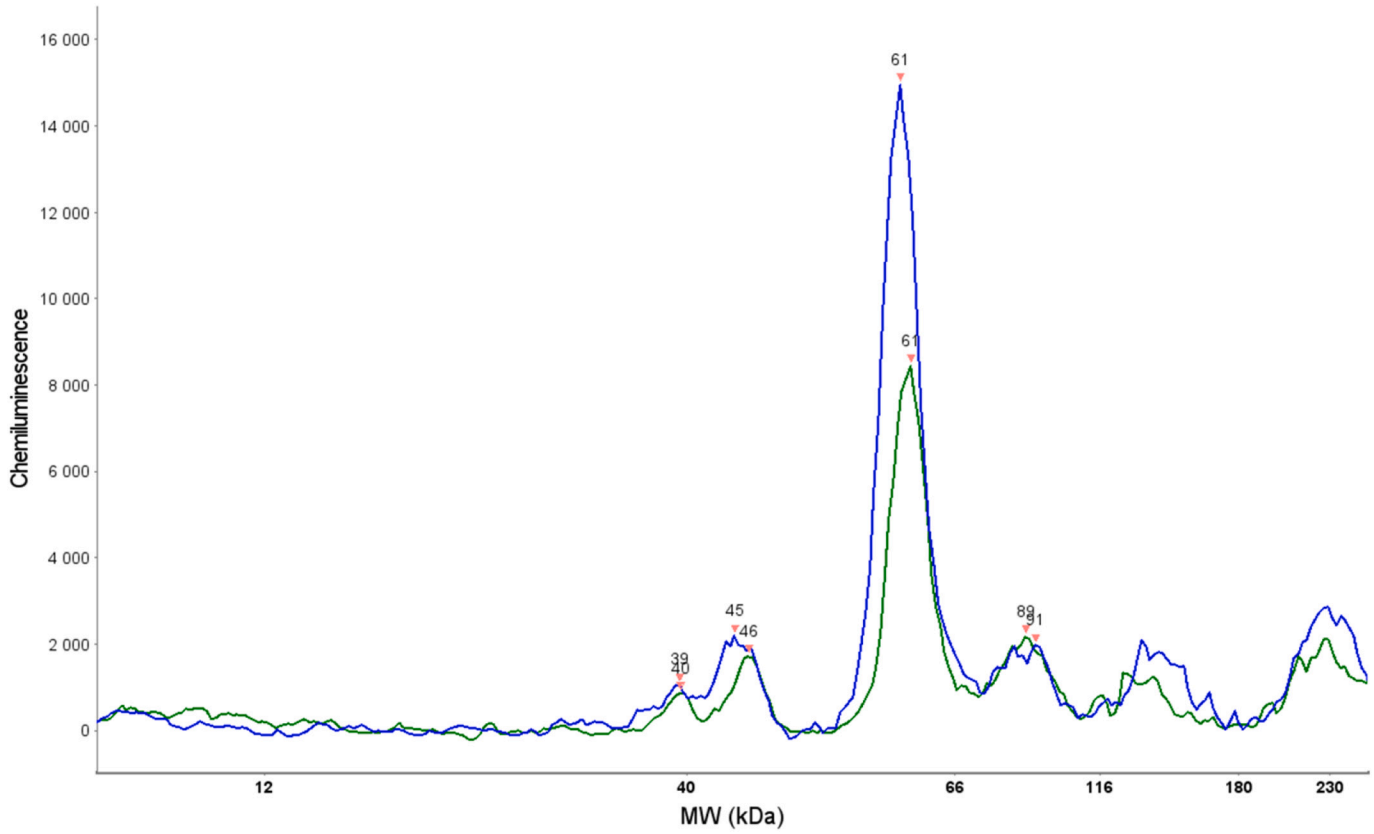

Fig. 3. 40 and 45 kDa ProNGF detected in OA chondrocytes by polyclonal antibody ab6199. The green curve shows the LPS stimulated sample and the blue curve shows the control. Peaks detected at 61 and around 90 kDa are not specific for NGF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quantification of NGF expression could not be performed. For information on the protein- and antibody concentrations used, see Supplementary table 2.

3.2. Wes controls

No unspecific binding was detected for the secondary antibodies, however, ab6199 showed unspecific binding to equine IgG at 31 and 59 kDa, and substantial unspecific binding to albumin at 61–64 kDa. MAB2562 showed no unspecific binding to either IgG or albumin (Fig. 1).

3.3. Nerve growth factor in Wes and western blot

When run with recombinant protein, peaks were detected by the polyclonal NGF antibody (ab6199) at 19, 32 and 46 kDa. The experiment was repeated with monoclonal proNGF specific antibody (MAB2562) at a higher protein concentration, and this time peaks were detected at 38 and 45 kDa, confirming that these peaks represented proforms of NGF (Fig. 2). In lysates, both antibodies detected consistent and specific peaks at 39/40 and 45/46 kDa (Figs. 3 and 4). No mature NGF could be detected with ab6199 at the concentrations used in this experiment. Peaks detected at other molecular weights could not be confirmed as NGF peaks as the ab6199 antibody showed various degrees of unspecific binding to albumin and IgG at corresponding molecular weights (Fig. 1). The same proNGF forms were excreted in both healthy and OA chondrocytes, and this was not altered by LPS stimulation. Bearing in mind that there was not saturation, no differences above the previously reported CV of 15% were seen in peak height between control cells or LPS stimulated cells, or between healthy and OA chondrocytes. Western blot confirmed the peaks at 40 and 45 kDa (Fig. 4).

4. Discussion

This is the first study to determine what specific forms of NGF are produced by chondrocytes. The results show that both healthy and osteoarthritic equine chondrocytes express NGF in 40 and 45 kDa proforms. Acute inflammation does not alter the expressed forms of NGF. The mature form of NGF (molecular weight of 13 kDa) could not be detected in the chondrocytes. These findings are in line with previous research in human patients with rheumatoid arthritis (Minnone et al., 2017b), where proNGF and not mNGF was predominant in synovial fluid and synoval fibroblasts. ProNGF with molecular weights around 40–45 kDa has been detected in dorsal root ganliga and colon of rats (Reinshagen et al., 2000), human and rodent cerebral cortex (Bruno and Cuello, 2006; Lobos et al., 2005), and commercial cell lines (Seidah et al., 1996). Moreover, not all cells in these studies expressed detectable levels of mature NGF which is in line with the results of Wes analysis of equine chondrocyte lysates.

Different cells have different NGF expression in regards to both the mNGF/proNGF expression and the molecular weights of the expressed proNGF. This is likely related to what arsenal of intracellular and extracellular cleavage enzymes are available (Bruno and Cuello, 2006; Seidah et al., 1996). Results from studies on one cell type can therefore not be extrapolated to all cells. In vivo research has shown that human chondrocytes express NGF, and that expression increases with worsening OA (Iannone et al., 2002). Osteoarthritic human and murine chondrocytes stimulated with IL-1β or visfatin in vitro showed increased NGF production (Pecchi et al., 2014) and TGF-β, another molecule in the downstream LPS signalling cascade has been shown to increase NGF production in murine, bovine and human chondrocytes (Blaney Davidson et al., 2015). However, in these studies mNGF was not distinguished from proNGF hence it is not known what NGF form was represented in

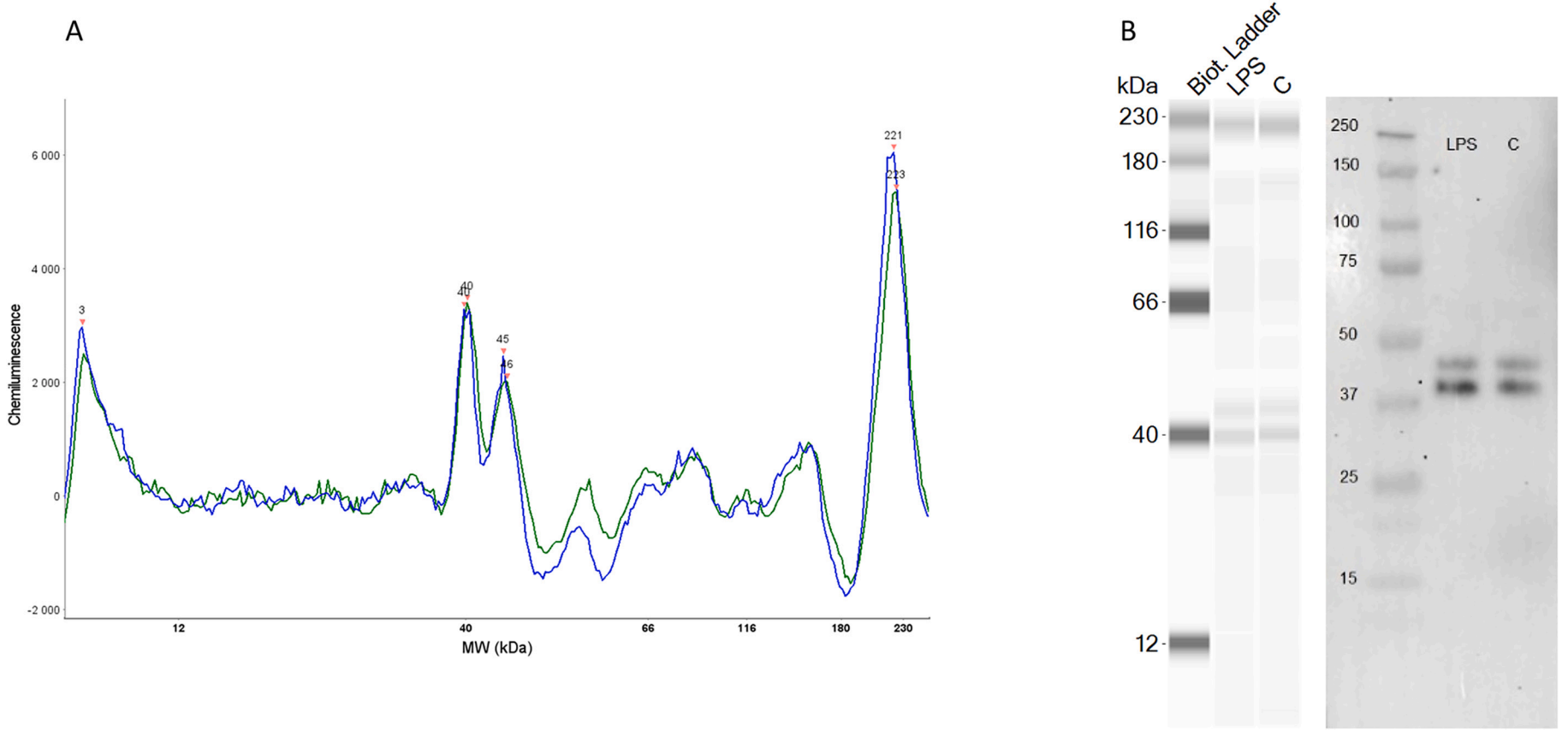

Fig. 4. ProNGF detected in OA chondrocytes by monoclonal antibody MAB2562.
A. Wes results: The green curve shows the LPS stimulated (LPS) sample and the blue curve shows the control (C). Peaks detected above 220 kDa are non-migrated proteins and are not considered significant.
B. Comparison of Wes data and western blot (far right). Both methods show proNGF at 40 and 45 kDa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the increase. Preliminary results from equine chondrocyte stimulation with IL-1β in our lab did not show changes in what proforms were expressed (unpublished data).

The ELISA method normally offers a quantitative analysis for comparison of sample concentrations of analytes. Most studies investigating NGF concentrations by ELISA have used antibodies that do not distinguish between proNGF and mNGF, but changes in the mNGF/proNGF ratio may be a better marker of disease and disease progression than changes in total NGF concentrations (Soligo et al., 2015). As most antibodies recognising mNGF will also have affinity for proNGF, antibodies specific for proNGF could offer a better alternative for ELISA. However, many different forms of proNGF have been described (Lee et al., 2001; Reinshagen et al., 2000; Soligo et al., 2015) and if proNGF antibodies are used they should preferably recognise the potential proforms in the sample of interest. Adding to these difficulties is that the NGF forms can reciprocally interfere in ELISA analysis, causing substantial variations in assay results depending on the mNGF/proNGF ratio (Malerba et al., 2016).

The Simple Western Systems are marketed as a quick and reproducible alternative to traditional western blots, with the additional advantage of being quantitative (https://www.proteinsimple.com/simple_western_overview.html?gclid=EAIaIQobChMI56nLzefq9gIVr4xoCR05qQ9XEAAYASAAEgLLsfD_BwE reference on March 29th 2022). As molecules are size separated, proNGF can be distinguished from mNGF. The method was tested for NGF analysis in equine chondrocyte lysates in this study. In our hands, the Simple Western was indeed quick and sample sparing. However, it did not allow for quantification as saturation of antibodies could not be achieved. One interesting possibility could be to perform a calibration curve in each run and determine concentrations based on this (Fourier et al., 2019). That method would significantly decrease the number of samples that can be compared within each run and it would also require a recombinant or purified source of appropriate forms of proNGF. Moreover, several attempts were made to analyse NGF in equine serum and synovial fluid on Wes but these experiments were unsuccessful due to substantial unspecific binding to albumin and IgG with all antibodies tested. Interestingly, unspecific binding of ab6199 to IgG was found to be both linear and saturable (data not shown).

The Wes method did show that equine chondrocytes in vitro produce 40 and 45 kDa proNGF, and the types of proforms expressed were not different between healthy and OA chondrocytes. Also, the proforms did not change when cells were subject to an acute inflammatory event by LPS stimulation. Galve-Roperh et al. (1997) showed that LPS at 10 ng/ml induced NGF production in rat astrocytes, but the maximal increase was seen with an LPS dose of 2 μg/ml which is considerably more than was used in the present study. In addition, varied effects are seen with different types and purities of LPS (Parusel et al., 2017). It is possible that the 10 ng/ml LPS dose used in this study was not sufficient to induce changes in NGF production in our model, but the model has previously been shown to increase OA chondrocyte intracellular Ca^{2+} release and expression of glutamate which indicates an inflammatory response (Skiöldebrand et al., 2019).

It is also possible that mNGF would have been detectable at a much higher protein concentration. Soligo et al. (2015) showed that mNGF could be detected in rat brain tissue when the protein load was increased from 20 to 100 μg. The concentration used in Wes (1 mg/ml) was within the linear range and was chosen based on the best performance for baseline and signal to noise ratio. Further increase of protein load up to 1.64 mg/ml did not improve peaks or reveal other proforms but it increased the baseline/background interference. The 9 μg protein load for western blot was the maximum dose that could be loaded equally for all samples when considering the total protein content. The samples used in the present study were pure chondrocyte lysates, likely providing a more concentrated source of analyte than whole tissue lysates.

5. Conclusion

Equine chondrocytes produce 40 and 45 kDa proNGF. If mNGF is produced, the amount is considerably less as no mNGF was detected either in Wes or western blot at the protein concentrations analysed. The finding of only proNGF may have implications for development of future therapies for OA-related pain, as monoclonal antibody therapies may in fact be better directed against the specific proforms produced by chondrocytes. Currently available anti-NGF therapy has an undetermined specificity for the different forms of NGF. In addition to this, the NGF receptor expression (TrkA and $p75^{NTR}$) and thereby the signalling pathways may also differ between disease stages (Kendall et al., 2022). Therefore, further definitions of NGF expression (mNGF/proNGF balance) and receptor expression, as well as more detailed definition of individual osteoarthritic disease stages may provide a key to determine what patients with OA would benefit from monoclonal NGF antibody therapy and what patients would be at increased risk of detrimental side effects.

Further studies on extracellular processing of NGF and the proNGF/mNGF ratio in health and OA disease are warranted and there is a need for developing quantitative methods such as ELISA that are specific for the 40 and 45 kDa proforms.

Declaration of Competing Interest

The authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.08.015.

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