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Quantitative top down mass spectrometry identifies proteoforms differentially released during mechanical stimulation of mouse skin

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Abstract

Mechanotransduction refers to the processes whereby mechanical stimuli are converted into electrochemical signals that allow for the sensation of our surrounding environment through touch. Despite its fundamental role in our daily lives, the molecular and cellular mechanisms of mechanotransduction are not yet well-defined. Previous data suggest that keratinocytes may release factors that activate or modulate cutaneous sensory neuron terminals, including small molecules, lipids, peptides, proteins, and oligosaccharides. This study presents a first step towards identifying soluble mediators of keratinocyte-sensory neuron communication by evaluating the potential for top down mass spectrometry to identify proteoforms released during one minute of mechanical stimulation of mouse skin from naïve animals. Overall, this study identified 47 proteoforms in the secretome of mouse hind paw skin, of which 14 were differentially released during mechanical stimulation, and includes proteins with known and previously unknown relevance to mechanotransduction. Finally, this study outlines a bioinformatic workflow that merges output from two complementary analysis platforms for top down data and demonstrates the utility of this workflow for integrating quantitative and qualitative data.

Graphical Abstract

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Supporting Information

The following supporting information is available free of charge at ACS website: https://urldefense.proofpoint.com/v2/url?u=http-3A__pubs.acs.org&d=DwlCaQ&c=aFamLAsxMIDYjNglYHTMV0iqFn3z4pVFYPQkjgspw4Y&r=9IRmljelwkoD3yAjnCgRA&m=kXOIlcADkpn0SedfPioiT0C9xDIZkz5NUzgqRdbXHbM&s=_5_N7pN2GEI1Rt3b2vR22Mttxhzaxr5MyCYFKesmoA&e=

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Keywords

Mechanotransduction; keratinocytes; top down proteomics; label free quantitation; nociceptor; sensory neuron; touch; primary afferent; proteoforms

Introduction

Mechanotransduction refers to the processes whereby mechanical stimuli are converted into electrochemical signals that allow for the sensation of our surrounding environment through skin touch. The molecular and cellular mechanisms of mechanotransduction in skin play fundamental roles in our daily lives on a moment to moment basis: they allow us to perceive tactile stimuli from pleasant stroke to noxious insult, and they allow us to appropriately grip objects and accurately move our limbs. However, the mechanistic details of these vital processes are not yet well-defined. As our largest organ, the skin provides a vast receptive area for a plethora of diverse tactile inputs into the nervous system. Mechanical forces impact the skin and, via neural circuitry through the central nervous system, inform the brain about our physical environment, which in turn, guides our actions. Sensory neurons in the skin have long been assumed to be the major, if not the sole site for mechanotransduction. However, anatomically, sensory neurons are not the initial point of contact for mechanical stimuli to the skin. Rather, environmental stimuli first contact the outer epidermis. Ninety-five percent of the epidermis is comprised of keratinocytes, which are proximal to cutaneous sensory terminals, and synapse-like contacts with sensory neurons have been suggested^{1–2}. Although our understanding of the molecular events involved in mechanotransduction is incomplete, a landmark study in 2010 identified PIEZO1 and PIEZO2 as key ion channels that are directly mechanically sensitive and underlie the fast-activating and inactivating mechanical cation currents in many mammalian cell types³. Subsequently, PIEZO2 was determined to be the key ion channel underlying mechanical currents and responsiveness in rapidly activating neurons that sense light touch in the skin⁴.

Several studies have shown that non-neuronal Merkel cells, which comprise 3–6% of the glabrous or the hairy skin, also express PIEZO2 which is essential for Merkel cell mechanical sensitivity^{5–7}. Importantly, recent studies have demonstrated that Merkel cells are essential in modulating the response of slowly adapting type 1 A β -fiber sensory afferent

terminals to sustained mechanical force and that mechanical force is detected via collaboration between Merkel cells and their innervating sensory afferent terminals^{8–10}. Furthermore, it has been suggested that Merkel discs utilize serotonin to convey tactile signals from Merkel cells to whisker afferent endings in the epidermis¹¹, thereby revealing a potential signaling mechanism between these two cell types. These findings introduced the concept that other non-neuronal cells in skin may also participate in modulating or tuning the response of cutaneous sensory neurons to tactile stimuli. Indeed, a recent study showed that selective optogenetic activation of keratinocytes is sufficient to drive action potentials in cutaneous sensory neurons¹². Conversely, optogenetic inhibition via stimulation of halorhodopsin in keratinocytes, which hyperpolarizes keratinocytes, is sufficient to dampen action potential firing in sensory neurons¹². Further, our recent study showed that keratinocytes play a vital role in the detection of innocuous and noxious touch¹³. Mechanistically, keratinocytes release ATP upon innocuous and noxious touch, which in turn activates P2X4 receptors on sensory neurons, thereby causing action potential firing in the neurons leading to touch detection¹³. Collectively, these data demonstrate that communication occurs between these keratinocytes and sensory neurons in response to tactile stimuli. However, inhibition of the ATP-P2X4 signaling cascade did not completely abolish mechanical behavioral responses in these animals¹³. This is likely due to the expression of mechanically sensitive ion channels on sensory neurons^{3, 14–15} and the possibility that keratinocytes may release other additional factors that activate or modulate cutaneous sensory nerve terminals in response to mechanical stimulation. Although keratinocytes are known to be capable of releasing neurotransmitters and neuropeptide signaling molecules such as ATP^{16–17}, calcitonin gene-related peptide β ¹⁸, acetylcholine¹⁹, glutamate²⁰, epinephrine²¹, neurotrophic growth factors²², and cytokines²³, the signaling factors that are specifically released from intact skin in response to mechanical stimulation have not been comprehensively investigated.

Secreted or released factors involved in mechanotransduction may include soluble or microvesicle-bound small molecules, lipids, peptides, proteins, and oligosaccharides, among others. Therefore, a comprehensive investigation into the molecular signaling mechanisms involved in mechanotransduction will require identification, characterization, and quantitation of each of these molecular species. Mass spectrometry (MS) is well-suited for analyzing each of these molecular classes and is therefore expected to play a central role in revealing released factors involved in mechanotransduction signaling. Here, we utilized a top down MS approach to characterize proteoforms, which are collectively defined by a protein's amino acid sequence, including cleavage and splice variants, and post-translational modifications, to provide precise whole-protein level information^{24–25}. As a first step towards comprehensively defining the molecular basis of mechanotransduction, the focus of this study was to determine whether it is possible to identify proteoforms released during one minute of mechanical stimulation of skin taken from naïve mice, using a minimalistic sample preparation approach. Overall, by merging outputs from two complementary analysis platforms for top down data, BioPharma Finder and ProSightPD, this study demonstrates that top down MS can identify proteoforms differentially released during mechanical stimulation of the skin and reveals proteoforms previously not reported to be involved in mechanotransduction.

Material and Methods

Animals and mechanical stimulation of glabrous hind paw skin

An overview of the experimental workflow is shown in Figure 1. Adult male C57BL/6 mice at least 8 weeks of age (Jackson Laboratories, Bar Harbor, ME) were used. Mice were provided with food and water *ad libitum* and were housed in a 14:10 hour light:dark cycle on Sani-Chips® and aspen wood chip bedding (P.J. Murphy Forest and Products, New Jersey) with Enviro-dri® nesting material (Shepherd Specialty Papers, Michigan). All animals were maintained with experimental protocols approved by the Medical College of Wisconsin and performed in accordance with the Institutional Animal Care and Use Committee (approval #: 0383). Animals were briefly anesthetized via inhaled isoflurane and then sacrificed via cervical dislocation in accordance with approved institutional protocols. For each mouse (n=6), the glabrous skin of both hind paws was cleaned with 70% ethanol, dissected away from muscle and tendons, cut into three equal sized sections approximately 3 × 3 mm. Sampling was randomized across anatomical distributions. Specifically, two sections (toe, middle or heel area) from each paw were assigned to one experimental group and the third was assigned to the other group; the opposite was done with the sections from the second paw. For example, if the toe and middle sections were assigned to the first group and the heel to the second group for the first paw, the heel was assigned to the first group and the toe and middle sections to the second group for the second paw. This allowed for equal anatomical distributions where each stimulation group contained one section from the toe area, middle section and heel area from each mouse. Each hind paw section was immediately placed with the stratum corneum facing down into individual wells of a 96-well plate filled with 80 µl of physiological buffer containing (in mM): 123 NaCl, 3.5 KCl, 2.0 CaCl₂, 0.7 MgSO₄, 1.7 NaH₂PO₄, 5.5 glucose, 7.5 sucrose, 9.5 sodium gluconate, and 10 HEPES, pH 7.45 ± 0.05 or calcium-only buffer (2.0 mM aqueous CaCl₂, pH 7.45 ± 0.05). The skin section was then immediately and repeatedly mechanically stimulated (at roughly 2 Hz) for 1 minute with a 20 mN von Frey filament (*i.e.* mechanically stimulated) or left in the solution for the same time without mechanical probing (*i.e.* unstimulated). Importantly, the 20 mN force applied is on the lower ascending slope for the amount of force typically applied in *ex vivo* skin nerve preparations where the maximal stimulation is 200–300 mN, forces for which action potentials are routinely recorded from the embedded sensory terminals^{26–27}. Therefore, this stimulation is unlikely to result in tissue damage that would induce cellular apoptosis. Subsequently, the solution was removed from each well and the samples were kept on ice until MS analyses were performed. For each mouse, three biological replicates (*i.e.* separate hind paw skin sections) were prepared for each condition (mechanically stimulated vs. unstimulated). From each biological replicate, 10 µl was removed and used for protein quantitation using the Qubit fluorometer (Thermo Fisher Scientific, San Jose, CA) per manufacturer's instructions. The remaining sample volume was adjusted with physiological buffer such that total protein concentration among three technical replicates within a single experimental condition for a single mouse were equivalent. One mouse, totaling 6 samples (3 mechanically stimulated + 3 unstimulated), was prepared per day to minimize the time each sample was stored at 4°C prior to MS analysis.

Trypan blue staining of whole skin

Trypan blue staining was performed to test for cellular apoptosis. Whole skin from the glabrous hind paw was dissected from 4 euthanized mice as described above. Sections were randomly assigned in equal anatomical distribution to the three different groups (unstimulated, stimulated with 20 mN von Frey Filament and positive control with 18 g needle stimulation) similar to the sampling procedure described above such that the different sections from the same paw did not always receive the same treatment. For 1 min each, skin sections were either unstimulated, stimulated with the 20 mN von Frey Filament or stimulated with the 18g needle at approximately 2 Hz in the physiological buffer. The needle stimulation was performed so that the needle punctured the skin to guarantee damage, thus serving as a positive control. Subsequently, the skin was placed in a 50:50 mixture of Trypan Blue (Sigma-Aldrich, St Louis MO) and physiological buffer for 30 seconds. The skin was quickly rinsed by dipping in physiological buffer prior to imaging using a Nikon SMZ1500 microscope.

Top down mass spectrometry

Samples were analyzed by liquid chromatography MS/MS using a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) in line with a Q Exactive Hybrid Quadrupole Orbitrap MS (Thermo Fisher Scientific). All technical details are provided in Table S1. For each injection, 20 μ l neat sample (14–71 ng/ μ l) was loaded using full-loop injection mode onto a trap column (PepSwift Monolithic Trap 200 μ m ID x 5 mm, Monolithic PS-DVB (Thermo Fisher Scientific)), and washed with mobile phase A (0.1% (v/v) formic acid in water) for 5 min at 8 μ l/min before elution onto a PLRP-S PicoChip column (75 μ m ID, 10.5 cm, 5 μ m, 1000Å (New Objective, Woburn, MA)). Proteins were separated using a linear gradient from 2.0% mobile phase B (80% acetonitrile, 0.1% (v/v) formic acid in water) to 60.0% B in 20 minutes at a flow rate of 300 nl/min. Proteins were analyzed using a top down strategy with in-source CID set at 15 eV, scan range of 500 – 2000 m/z , and four microscans were acquired for each spectrum. MS1 spectra were acquired using a target setting of 1×10^6 ions, an accumulation time of 50 ms, and scanned at a resolution of 140,000 (at 200 m/z). Each MS1 scan was followed by HCD fragmentation of the two most abundant precursor ions with a charge state of +4 or greater and dynamic exclusion set to 20 s. MS2 spectra were acquired using a target setting of 1×10^6 ions, an accumulation time of 150 ms, scanned at a resolution of 70,000 (at 200 m/z), isolation window set to 5.0 m/z , and normalized collision energy set to 21. For the initial test comparison of the two sample buffer solutions, a single injection was performed for each sample. For the subsequent analyses of the physiological buffer, three replicate injections were performed. All MS data files are publicly available at MassIVE (MSV000082059; massive.ucsd.edu).

Data Analysis – Identification

Data were analyzed using ProSightPD 1.1 (Proteinaceous, Evanston, IL)) as a plug-in node within Proteome Discoverer 2.2 (Thermo Fisher Scientific). Data were searched against *Mus musculus* (mouse) proteoform database (created 7/2016; top down complex database warehouse from Proteinaceous, 36,596 proteins, 3,259,647 proteoforms) using a two-tier search workflow as described in detail in Table S1. Briefly, tier one consisted of an absolute

mass search with 2.2 Da precursor mass tolerance and 10 ppm fragment mass tolerance. Tier two consisted of a biomarker search with 10 ppm no-enzyme precursor mass tolerance and 10 ppm fragment mass tolerance. Results were filtered to include peptide spectrum matches (PSM) that were assigned a search engine rank of 1, -Log E-value ≥ 4 and more than 4 fragment ions. All proteoforms reported were identified by at least 10 PSMs and observed in at least three mice within a single experimental condition either based on ProSightPD (proteoform identified) or BioPharma Finder (precursor mass detected; see below). To match components detected in BioPharma Finder with proteoforms identified by ProSightPD, features were required to match within 0.1 Da to a PSM assigned to specific proteoform by ProSightPD and within a 1 min window (± 30 sec) around the average retention time reported by ProSightPD. In some cases, proteoforms identified in ProSightPD could not be matched with certainty to specific features reported by BioPharma Finder and are annotated as 'ambiguous' in Table S2.

Data Analysis – Relative Quantitation

Data were analyzed with BioPharma Finder version 2.0 (Thermo Fisher Scientific). An intact protein workflow processing method was created for Sliding Window Deconvolution using the Xtract deconvolution algorithm. Chromatograms were scanned over full retention time range with charge state range of +4 to +40, sliding windows merge tolerance for components was 30 ppm, multiconsensus component merge tolerance was 10ppm, and the minimum number of detected charge states to produce a component were designated as 3. For each comparison set, the monoisotopic masses of components, their sum intensities for all charge states detected, charge state distribution, retention time range, and the number of raw files found to contain those with corresponding intensities were exported to Excel for further processing. The average intensity for each feature was calculated per hind paw section per mouse. Non-detected features were assigned a value of zero. Average intensity values were normalized by a scaling factor based on total protein quantity and the statistical significance between treatment groups and among mice was assessed by a paired two-way ANOVA followed by *Sidak's* multiple comparison tests. A paired two-way ANOVA was chosen because multiple mechanically stimulated and unstimulated samples were obtained from the same animal. In this way, the stimulated and unstimulated sections for each mouse were compared as groups, treating the three sections in each group as independent measurements. Statistical analysis was only performed for features which could be matched to proteoforms identified by ProSightPD and which were identified in at least 3 mice. The stimulated to unstimulated ratio reported in Table 2 was calculated on a per-mouse basis taking the average normalized intensity of the groups of stimulated and unstimulated sections.

Results

The method used to mechanically stimulate the glabrous hind paw skin in this study is similar to the *ex vivo* glabrous skin-nerve preparations we routinely utilize for electrophysiological functional assessment of cutaneous terminals of primary afferent fibers 13, 26, 28. The isolated glabrous hind paw skin was mechanically stimulated with graded von Frey filaments. Modest force (20 mN) was used to avoid tissue damage and this force is in

the lower half of the range we use for skin-nerve electrophysiological recordings (2–300 mN)^{13, 29}. Further, we routinely observe that when skin-nerve preparations are maintained *ex vivo* in physiological buffer, the nerve terminals retain their physiological viability and response properties for at least six hours. Here, trypan blue staining of whole skin was used as an estimate of cellular apoptosis. Trypan blue is routinely used as a stain to assess cell viability via a dye exclusion test; as such, viable cells should not take up the impermeable trypan blue dye. Upon visual inspection of the skin sections, no significant differences were found between the unstimulated and the 20 mN (2 Hz for 1 min) mechanically stimulated group (Figure S1 A and B). However, the positive control (Figure S1C, 18g needle stimulation) showed significantly more blue staining than either the unstimulated sections or the sections stimulated with the 20 mN von Frey filament (Figure S1 A-C). Overall, our previous findings of viable skin-nerve preparations that utilize a range of mechanical forces,^{13, 26, 28} and the trypan blue staining results from the current study suggest that there is limited cell death using this approach. Altogether, these observations support the use of this method for studies aimed at identifying mechanically-released proteins. To minimize technical artifacts that may result from drying under vacuum, freezing, or extensive sample handling, we tested whether the analysis of neat samples would yield sufficient protein content to provide interpretable top down MS data and began MS analyses immediately after sample collection. Although the physiological buffer is expected to yield more biologically relevant data than the calcium buffer, it was initially unclear whether the physiological buffer components would adversely affect MS performance. Therefore, preliminary experiments compared mechanically stimulated and unstimulated samples from both buffer types. In these analyses, the physiological buffer did not appear to interfere with the identification of proteoforms as compared to the calcium-only buffer (data not shown). Moreover, most proteins identified as putatively unique to the mechanically stimulated condition as compared to the unstimulated samples for the calcium-only buffer (data not shown) were also observed in the mechanically stimulated condition for the physiological buffer. Therefore, all remaining analyses were conducted in the physiological buffer because it is a more biologically relevant matrix. Finally, we considered whether sample stability at 4°C would present challenges. Although samples for a single mouse were collected simultaneously, MS data were acquired in series with ~60 min separating each injection. Importantly, we did not observe any significant trends that tracked with the order in which samples were analyzed that would indicate that samples run later in the queue were missing proteins or contained degraded proteoforms compared to those run earlier. Altogether, these observations support the utility of the approach for revealing putative differences related to the experimental condition as opposed to technical artifacts.

Overall, 47 proteoforms were identified by ProsightPD in the comparison of mechanically stimulated vs unstimulated groups (Table 1; Table S2, Figures S2-S3). The majority (90%) of proteins identified here are annotated as extracellular or extracellular exosome-related (UniProt). This finding supports the utility of this approach for identifying secreted proteins, although some likely contaminants are present (*e.g.* fragments of hemoglobin, albumin). Thirty proteoforms represent 99% coverage of the intact protein (Table 1), suggesting that proteolytic cleavage resulting from sample handling was minimal. For those proteoforms that represent cleavage products, most are consistent with known biology. For example,

filaggrin plays a pivotal role in skin barrier function³⁰, and the cleavage products for filaggrin observed here in both unstimulated and mechanically stimulated samples are consistent with the extensive proteolytic degradation of filaggrin that occurs during epidermal terminal differentiation³⁰.

Details of the quantitative workflow are shown in Figure 2. The mechanically stimulated samples routinely yielded a higher total protein content (2–3 fold) compared to unstimulated samples; therefore, to avoid over-diluting the mechanically stimulated samples and to account for the fact that each mouse was processed separately, total protein content was normalized within a treatment group (mechanically stimulated or unstimulated) for each animal. To account for this during data analysis, the summed intensity for a proteoform was normalized by a scaling factor reflective of differences in total protein quantity. Overall, this strategy revealed 11 proteoforms that were not statistically significantly different between the treatment groups (Table 2) including those that are likely background contaminants (*e.g.* albumin, hemoglobin, copper transport protein ATOX1).

Seven proteoforms were either exclusively detected in the mechanically stimulated group or were significantly more abundant in the mechanically stimulated compared to the unstimulated group. These include proteoforms 8103.21 and 11048.49 from High mobility group protein B1 (HMGB1) and Protein S100-A10 (S100A10), respectively, which were identified uniquely in the mechanically stimulated group by ProSightPD (Table 1). Quantitative analyses from BioPharma Finder were consistent with the identification results, where deconvoluted monoisotopic MS1 values were only detected in the mechanically stimulated group (Figure 3A-B). Results for proteoform 6821.69 from Apolipoprotein C-I (APOC1) were similarly consistent between the two platforms, as it was identified uniquely in the mechanically stimulated group by ProSightPD and quantitation in BioPharma Finder found low level detection only in unstimulated hind paw sections of a single mouse (Figure 3C). Proteoform 8704.2 from Apolipoprotein A-II (APOA2) was more abundant in the mechanically stimulated condition across the five mice in which it was detected by BioPharma Finder (Figure 3D), consistent with more PSMs identified in mechanically stimulated (n=213) than in unstimulated (n=142) samples by ProSightPD (Table 1). Finally, seven proteoforms were less abundant in the mechanically stimulated samples, including three proteoforms of Calmodulin-4 (CALM4; 8950.57, 9363.77, 16668.42; Figure 4A; Table 2) which represent the full length and cleaved forms (Figure 4C), respectively, and two proteoforms of Acyl-CoA-Binding protein (DBI; 9906.12, 9922.10; Table 2, Figure 4B), which represent unmodified full-length protein and modified truncated protein (Figure 4C), respectively.

Overall, these analyses suggest that even with limited protein content (280–1420 ng per MS injection) and short duration (1 min) of mechanical stimulation of the skin, top down MS can reveal proteoforms that are differentially released with mechanical stimulation. Although there are variations in the summed peak intensity for individual proteoforms among animals, which is consistent with the fact that samples among animals were not normalized to each other due to their collection and analysis on different days, the trends in the relationship between mechanically stimulated and unstimulated among animals are largely consistent for the proteoforms quantified here. Altogether, these analyses highlight

the power of integrating data between two platforms (ProSightPD and BioPharma Finder), as BioPharma Finder provides relative quantitation based on extracted ion chromatograms from multiple charge states but not proteoform identity, and ProSightPD provides identity and the number of PSMs observed.

Discussion and Conclusions

Although significant progress has been made in identifying ion channels involved in mechanotransduction^{27, 31–33}, a detailed understanding of the full complement of molecules involved in signaling processes that occur between non-neuronal skin cells, such as keratinocytes and sensory neurons, is not yet established. Such information is important for advancing our understanding of the mechanisms that regulate baseline mechanosensory detection, as well as the mechanical sensitization that occurs with tissue injury or disease. Moreover, it is expected that precisely defining the molecules involved in cell-to-cell communication in the skin could lead to potentially novel topical analgesics or antipruritics. Keratinocytes have been shown to communicate with multiple subtypes of sensory neurons^{12–13, 34}. Different neuronal subtypes are thought to transmit different sensations; for example A β fibers generally transmit gentle touch, whereas C fibers generally transmit painful stimuli³⁵. Therefore, mechanistically defining keratinocyte to sensory neuron signaling may reveal mechanisms underlying transmission of innocuous and noxious mechanical stimuli during both acute pain and chronic pain settings, and during acute or chronic itch conditions. To address this knowledge gap, we applied top down MS to quantify and characterize proteoforms released by cutaneous mechanical stimulation of mouse skin. MS is well-suited to the analysis of factors that are rapidly released during mechanotransduction as it can directly identify and quantify proteins with very low limits of detection without antibodies or *a priori* knowledge of the molecular composition.

Among the proteoforms identified as uniquely present or more abundant in the mechanically stimulated samples, HMGB1, APOA2, APOC1, and S100A10 are particularly interesting due to their known biological relevance to skin biology, including pain. HMGB1 can be released from keratinocytes, and the injection of complete HMGB1 protein into the hind paw of mice causes acute nociceptive behaviors *in vivo*^{36–37}. Although HMGB1 is known for its proinflammatory functions, it has also been shown to promote skin wound healing³⁸. Our MS analysis revealed that specifically the B-box domain of HMGB1 (residues 90–161), which contains the cytokine activity and toll-like receptor binding domain, was found in the mechanically stimulated condition. The B-box domain has been demonstrated to recapitulate the cytokine activity of full length HMGB1 where it can efficiently induce the secretion of proinflammatory cytokines from macrophages^{39–40}. Additionally, the B-box domain of HMGB1 has been shown to induce marked derangements in gastrointestinal mucosal barrier function in mice^{40–41}, which further suggests a role for HMGB1 B-box domain in inducing functional alterations in epithelial cells. Another protein found in higher abundance in the stimulated group was S100A10. S100A10 is found in both the cytoplasm and periphery of basal and suprabasal keratinocytes⁴². In normal epidermis it is localized to the nucleus and cytoplasm, however, in psoriatic tissue, its localization is increased in the plasma membrane⁴³. S100A10 also interacts with ion channels such as Nav1.8, which are involved in neuropathic pain and are expressed in keratinocytes and nociceptive sensory neurons^{44–46}.

Therefore, it is possible that these ion channels can be modulated through the interaction with S100A10, which tethers them to the cell membranes. In summary, the crucial role of S100A10 in keratinocyte differentiation⁴⁷ and its interactions at the plasma membrane are consistent with the increased release upon mechanical stimulation observed here. Finally, APOA2 and APOC1 were more abundant in the stimulated samples. Although APOC1 is perhaps better recognized as an important lipoprotein binding protein in circulating blood, APOC1 is also normally expressed in the skin⁴⁸. Importantly, mice that lack APOC1 exhibit cutaneous abnormalities such as dry scaly skin with hair loss, whereas mice that express human APOC1 exhibit dermatitis and atrophic sebaceous glands lacking sebum^{49–51}. These data suggest that APOC1 may have a crucial role in lipid homeostasis of the skin that is consistent with its role in binding and transporting lipids among various tissues⁵². APOC1 has also been shown to be upregulated after mechanical massage of femoral adipose tissue⁵³. This is consistent with our results where APOC1 abundance was increased in samples after mechanical stimulation. Two APOA2 proteoforms were identified, and while both were observed by more PSMs in the stimulated, only the proteoform with pyrrolidone carboxylic acid at the N-terminus reached a level of statistical significance in the quantitation. This modification can protect proteins from degradation by amniopeptidases, is commonly observed in secreted proteins, and has been reported on human APOA2⁵⁴. APOA2 is involved in mediating cholesterol efflux from cell membranes⁵⁵ and reductions in plasma membrane cholesterol dampen mechanotransduction responses⁵⁶. Lipids such as cholesterol-enriched membrane domains have been suggested to play roles in the mechanotransduction process⁵⁶. Therefore, APOA2 may play a role in mechanotransduction processes by modifying membrane cholesterol content. Additionally, APOA2 is expressed in basal cells in the epidermis and APOA2 has been linked to cell proliferation and differentiation⁵⁷. Altogether, whereas, previously described functions of HMGB1, S100A10, APOC1 and APOA2 have suggested that they have important roles in epidermal physiology, our study is the first to directly link these proteins to mechanotransduction through their release upon mechanical stimulation of skin.

Our study also identified several proteoforms that were less abundant in the secretome after mechanical stimulation. This includes two proteoforms of Acyl-CoA-binding protein abbreviated as ACBP and also known as diazepam binding inhibitor (DBI). One proteoform of DBI is missing the last two C-terminal amino acids and contains both malonyl-lysine and succinyl-lysine modifications. While these modifications have recently been implicated as important regulators of metabolism, our knowledge regarding their biological significance is still in its infancy^{58–60}. Malonyl-lysine and succinyllysine modifications induce dramatic structural changes on lysine residues due to their large size and they frequently occur together⁶¹. Our data are consistent with these previous observations, however, in the context of DBI proteoforms, the biological consequence of these modifications is yet unknown. Generally, DBI is a negative allosteric modulator of GABA_A receptors that play a critical role in skin barrier homeostasis in keratinocytes^{62–63}. Skin tissue injury has been suggested to cause the depolarization of keratinocytes and prevent wound healing. In contrast, repolarizing keratinocytes via GABA_A-like receptors could be crucial in promoting wound healing through the fusion of the lamellar body and cell membrane⁶². Moreover, DBI has been shown to have anti-nociceptive effects in the central nervous system⁶⁴ and can be

released through an unconventional secretion mechanism in yeast⁶⁵ where DBI is secreted despite its absence of a canonical signal peptide. It is possible that DBI is endogenously present in the extracellular space of the skin, such that in absence of stimulation, DBI normally inhibits diazepam from binding to GABA_A receptors, akin to its action in the brain⁶⁶. Upon mechanical stimulation of the skin, DBI could be degraded or prevented from acting on GABA receptors and thereby, allow for the disinhibition of GABA_A receptors.

Alternatively, DBI has also been shown to regulate lipid metabolism. DBI knockout animals have been shown to have a >50% decrease in non-esterified very long chain fatty acid skin content as compared to wild type mice, and show distinct skin and fur phenotypes that are characterized by greasy fur and coat color changes that develop into alopecia with age⁶⁷. This suggests that DBI is important in incorporating very long chain acyl-CoA esters in complex lipids into the epidermis, and therefore may be necessary for normal skin barrier function. It is possible that its secretion is regulated by interactions with intracellular acyl-CoA-esters^{68–69}, thereby preventing secretion or alternatively interfering with their detection by this MS strategy.

Finally, three proteoforms of CALM4 were found to be less abundant in the stimulated samples. CALM4 is a calcium binding protein strongly expressed in subbasal keratinocytes and plays a role in restoring epidermal calcium gradient during wound healing and barrier formation^{70–71}. Interestingly, the subcellular localization of CALM4 in keratinocytes is linked to intracellular calcium concentrations and during acute barrier disruption where intracellular calcium is increased, CALM4 localizes to the nucleus⁷¹. Keratinocytes have been shown to respond to mechanical stimulation and allow calcium influx from the extracellular space⁷². Therefore, it is possible that upon mechanical stimulation of the skin, CALM4 is less available for secretion due to changes in intracellular calcium and its translocation to the nucleus. Additionally, intracellular calmodulin has also been shown to inhibit several ion channels upon binding, such as NMDA receptors, voltage gated sodium channels and calcium channels^{73–76}. Several ion channels, such as Transient Receptor Potential (TRP) channel family members (*i.e.* TRPC6 and TRPV1), have been shown to have a prominent overlap between phosphoinositides and calmodulin-binding sites⁷⁷. It is possible that the mechanical stimulation of the skin alters the phosphoinositide composition in keratinocyte or sensory neuron cell membranes within the skin. This could lead to the release of the inhibitory calmodulin-ion channel interaction, allowing ion channel activation. Overall, while it is not yet clear why these proteoforms were detected in lower abundance in the stimulated samples, their known functional roles and interactions with other molecules largely support several possibilities, including the mechanically-induced release of enzymes that degrade these proteins, or the stimulation of a signaling cascade that leads to the sequestration or degradation of these proteoforms upon mechanical stimulation.

Beyond the quantitative view of these data, one advantage of top down MS is the preservation of the relationship among amino acid sequence, cleavage sites, and post-translational modifications. Examples of where these proteoform analyses provide specific whole-protein level information that would be more challenging to obtain through a bottom-up strategy include the specific detection of the B-Box of HMGB1, two proteoforms of B2M which differ by a single amino acid substitution, unmodified full-length and modified

truncated DBI, cleavage products of CALM4, and CALM1 proteoforms containing either acetylation or trimethylation. Although the biological function of these modified proteoforms is not yet known, this quantitative discovery approach sets the stage for future studies aimed at precisely relating whole-level protein information to mechanotransduction.

While the method used here establishes the utility of top down MS for discovering novel factors involved in mechanotransduction, there are limitations. From a technical perspective, there is a preference for identification of proteoforms <25 kDa using the Q Exactive MS and fragmentation is limited to higher energy collision dissociation (HCD); however, these limitations can be overcome with more advanced instrumentation^{78–79}. Moreover, depth of coverage may be increased if a deglycosylation step prior to MS were performed, as many secreted proteins are N-glycosylated and this post-translational modification can complicate top down MS analysis. From a biological perspective, it is not possible to attribute the proteins identified here to their cellular origin, as skin contains not only keratinocytes, but other cell types including Merkel cells, fibroblasts, mast cells, dendritic cells, Langerhans cells, and sensory neurons.

Despite these limitations, these proof-of-concept data demonstrate that this approach can rapidly identify targets of interest for future studies that could include comparisons among larger sampling groups, ranges of stimulation intensities, variations in type of force applied (*e.g.* dynamic versus static), and furthermore, determine whether a tissue injury model would induce the release of other novel molecules or increase the release of the molecules identified in baseline non-*in vivo*-injured tissue. In conclusion, this proof-of-concept study shows that analysis of supernatant from mechanically stimulated naïve mouse skin by top down MS can reveal proteoforms not previously described as having a role in mechanotransduction and establishes a baseline of the proteome released from skin in response to tactile stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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of each sample were analyzed using a Q Exactive MS, yielding 9 mechanically stimulated and unstimulated replicates per mouse. The resulting 54 RAW files were analyzed separately by two platforms (ProsightPD and BioPharma Finder) and data were subsequently manually integrated by matching retention time and mass to integrate quantitative data for features identified in BioPharma Finder with proteoforms identified by ProsightPD.

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Figure 2. Experimental workflow for quantitative analysis of top down data.

Raw peak data were extracted in BioPharma Finder and processed to calculate the average summed intensity for each extracted feature per hind paw section. Differences in the amount of injected protein were used to normalize the summed intensities. Hind paw sections from the same mouse were treated as paired for statistical analysis. Plots for proteoforms of interest in Figures 3–4 were generated to visualize the normalized summed peak intensities, wherein the data points represent individual hind paw sections per mouse. The mean for

sample groups (mechanically stimulated and unstimulated) are shown on a per mouse basis (horizontal bars) and across mice (listed at the top of each plot).

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Figure 3. Examples of proteoforms that are more abundant in the mechanically stimulated samples.

Plots display the normalized summed peak intensities for each proteoform wherein the data points represent individual hind paw sections per mouse for (A) HMGB1, (B) S100A10, (C) APOC1, and (D) APOA2. The means for sample groups (mechanically stimulated and unstimulated) are shown on a per mouse basis (horizontal bars) and across mice (listed at the top of each plot). Statistical significance is indicated and statistical comparisons are based on a paired two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. denotes a non-significant result). Supporting MS data for each proteoform are provided in Figure S2.

Figure 4. Examples of proteoforms that are less abundant in the mechanically stimulated samples.

Plots display the normalized summed peak intensities for (A) CALM4 and (B) DBI wherein the data points represent individual hind paw sections per mouse. The means for sample groups (mechanically stimulated and unstimulated) are shown on a per mouse basis (horizontal bars) and across mice (listed at the top of each plot). Statistical significance is indicated and statistical comparisons are based on a paired two-way ANOVA (* $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. denotes a non-significant result). (C) Visual representation of the observed proteoform sequences for Calmodulin-4 and Acetyl-CoA-

Binding Protein shown next to the theoretical masses (in Da). Supporting MS data for each proteoform are provided in Figure S3.

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Table 1.

Proteoforms identified in the comparison of mechanically stimulated vs. unstimulated samples. For each proteoform, the UniProt accession number, protein symbol, proteoform mass (Theoretical MH⁺ (Da)), number of peptide spectrum matches (PSM) observed across all 6 mice (+ mechanically stimulated; - unstimulated) are provided. The “sequence length” was calculated based on the fully processed form (*i.e.* after removal of signal peptide or initiator methionine). Posttranslational modifications and/or other notable features regarding the proteoform sequence observed are noted. For proteoforms that differ from the predicted full-length sequence by more than N- or C- terminal processing, the amino acid residues contained in the proteoform, based on the pre-processing sequence, are also noted. Additional MS data supporting this information are provided in Table S2.

UniProt	Protein Symbol	Mass (Da)	#PSM		Sequence Length	Modifications, Features, [Residues]
			-	+		
P07724	ALB	2791.43	72	98	4	N-terminally processed (signal peptide) [25–48]
P09813	APOA2	8704.20	142	213	99	N-terminally processed (signal peptide) N-term(2-pyrrolidone-5-carboxylic acid) Truncated last C-terminal amino acid
P09813	APOA2	8721.22	10	39	99	N-terminally processed (signal peptide) Truncated last C-terminal amino acid
P34928	APOC1	6821.69	0	22	99	N-terminally processed (signal peptide) Truncated first two N-terminal amino acids
O08997	ATOX1	7201.63	187	158	100	N-terminally processed (Met) Disulfide (C11-C14)
P01887	B2M	11634.70	65	101	100	N-terminally processed (signal peptide) Disulfide (C25-C80)
P01887	B2M	11635.65	40	54	100	N-terminally processed (signal peptide) Disulfide (C25-C80) K→E variant at K64
P0DP26	CALM1	16780.82	54	20	100	N-terminally processed (Met) N-term acetylation Acetylation at K94
P0DP26	CALM1	16780.86	112	45	100	N-terminally processed (Met) N-term acetylation Trimethylation at K115
Q9JM83	CALM4	8950.57	81	32	53	N-terminally processed (Met) N-term acetylation [2–80]
Q9JM83	CALM4	9363.77	208	165	55	N-terminally processed (Met) N-term acetylation [2–84]
Q9JM83	CALM4	16668.42	203	183	100	N-terminally processed (Met) N-term acetylation
Q9D6P8	CALML3	16602.79	75	38	100	N-terminally processed (Met) N-term acetylation
P62965	CRABP1	15451.73	5	15	100	N-terminally processed (Met)
Q62426	CSTB	11081.56	8	29	100	N-term acetylation
P31786	DBI	9906.12	451	468	100	N-terminally processed (Met) N-term acetylation
P31786	DBI	9922.03	12	0	98	N-terminally processed (Met) N-term acetylation Malonyl-Lysine (K54) Succinyl-Lysine (K60)
Q05816	FABP5	15039.37	315	413	100	N-terminally processed (Met) N-term acetylation
Q8CF02	FAM25C	5534.93	16	41	58	[38–89]
Q8CF02	FAM25C	9272.87	150	281	100	N-term acetylation

UniProt	Protein Symbol	Mass (Da)	#PSM		Sequence Length	Modifications, Features, [Residues]
			–	+		
P26883	FKBP1A	11785.07	42	25	100	N-terminally processed (Met)
P11088	FLG	2448.19	4	27	8	[47–74]
P11088	FLG	2604.29	9	37	8	[46–74]
P01942	HBA	3344.67	20	64	22	[2–33]
P01942	HBA	3880.08	8	27	25	[107–142]
P01942	HBA	6674.58	10	7	43	[82–142]
P01942	HBA	14972.74	388	414	100	N-terminally processed (Met)
P02088	HBB-B1	3636.99	12	10	24	[113–147]
P02088	HBB-B1	3736.06	54	54	25	[112–147]
P63158	HMGB1	8103.21	0	11	33	[90–161] (B-box domain)
P61979–1	HNRNPK	8249.42	33	41	19	[381–457]
Q64433	HSPE1	10867.84	36	110	100	N-terminally processed (Met) N-term acetylation
P34884	MIF	12366.14	60	30	100	N-terminally processed (Met)
P17742	PPIA	17829.82	10	5	100	N-terminally processed (Met)
P17742	PPIA	17871.83	18	6	100	N-terminally processed (Met) N-term acetylation
Q9D0J8	PTMS	11335.12	57	12	100	N-terminally processed (Met) N-term acetylation
P32848	PVALB	11835.07	24	5	100	N-terminally processed (Met) N-term acetylation
Q99P72–2	RTN4	7409.27	29	15	5	N-term acetylation [1–65]
P08207	S100A10	11048.49	0	50	100	N-terminally processed (Met)
P07091	S100A4	11625.72	23	5	100	N-terminally processed (Met) N-term acetylation
P14069	S100A6	9956.27	74	72	100	N-terminally processed (Met) N-term acetylation
P60897	SEM1	8184.54	19	35	100	N-terminally processed (Met) N-term acetylation
Q00897	SERPINA1D	4205.20	11	15	8	[378–413]
P61957	SUMO2	10514.21	26	18	97	N-terminally processed (Met) N-term acetylation Truncated last two C-terminal amino acids [2–93]
Q6ZWY8	TMSB10	4934.53	16	2	100	N-terminally processed (Met) N-term acetylation
P20065–2	TMSB4X	4961.49	111	132	100	N-terminally processed (Met) N-term acetylation
P62984	UBA52	8560.62	114	81	100	Fully processed [1–76], without 77–128 C-terminal 60S ribosomal protein L40

Table 2.
Summary of proteoform quantitation results from BioPharma Finder.

For each proteoform, the mass (Theoretical MH+ in Da), protein symbol, number of mice and hind paw sections in which it was detected by BioPharma Finder for each condition, ratio of S/U (mechanically stimulated/ unstimulated) per mouse, and statistical significance between treatment groups are provided. U = unstimulated; S = mechanically stimulated. Statistical comparisons were made between treatment (unstimulated versus mechanically stimulated) and among mice via a paired two-way ANOVA followed by a Sidaks post hoc test. *p < 0.05 **p < 0.01, *** p < 0.001, **** p < 0.0001, n.s. denotes a non-significant result. Proteoforms are grouped by statistical significance, where top section = not significantly different among groups, middle = more abundant in mechanically stimulated samples, bottom = less abundant in the mechanically stimulated samples.

		# of Mice Observed In				# of Sections Observed In				Ratio (S/U) per Mouse						
Theoretical Mass MH+	Protein Symbol	U	S			U	S			1	2	3	4	5	6	Significant Difference
Not statistically significant between groups																
2791.43	ALB	5	6			17	17			0.8	1.4	2.3	1.1	1.4	0.9	ns
7201.63	ATOX1	6	6			18	18			0.5	0.5	0.7	0.5	0.5	0.4	ns
11081.56	CSTB	6	4			17	11			0.7	0.5	0.7	U	0.3	U	ns
5534.92	FAM25C	2	6			8	14			S	S	1.4	1.3	S	0.5	ns
14972.73	HBA	6	6			18	18			0.2	0.5	0.5	0.6	0.8	1.8	ns
15039.37	HBA	6	6			17	18			1.4	1.1	1.1	0.4	0.7	0.2	ns
3736.06	HBB-B1	3	4			7	5			0.2	0.2	U	ND	0.3	S	ns
8249.42	HNRNPK	4	5			13	13			1.6	0.5	0.9	0.3	ND	0.4	ns
9956.27	S100A6	4	6			14	17			S	1.8	1.3	0.6	1.7	0.6	ns
11544.93	STFA3	4	3			9	6			0.6	0.4	0.2	U	ND	ND	ns
4961.49	TMSB4X	5	6			16	18			0.1	0.1	1.4	1.4	9.1	1.1	ns
More Abundant in Stimulated																
8704.20	APOA2	5	5			14	15			2.4	2.5	3.0	1.2	5.1	ND	****
6821.69	APOC1	0	6			0	14			S	S	S	20.4	S	S	****
9272.87	FAM25C	4	6			11	18			S	4.3	2.0	1.4	7.3	0.6	*
2604.29	FLG	0	6			0	12			S	S	S	S	S	S	*
8103.21	HMGBl	0	6			0	12			S	S	S	S	S	S	****
10867.84	HSPE1	3	6			10	18			5.6	15.2	1.1	1.5	S	1.5	**
11048.49	S100A10	0	6			0	9			S	S	S	S	S	S	**

		# of Mice Observed In		# of Sections Observed In		Ratio (S/U) per Mouse						
Theoretical Mass MH+	Protein Symbol	U	S	U	S	1	2	3	4	5	6	Significant Difference
Less Abundant in Stimulated												
8950.57	CALM4	5	5	15	10	0.4	0.1	0.5	0.3	0.1	U	**
9363.77	CALM4	6	6	18	18	0.3	0.2	0.5	0.3	0.3	0.2	**
16668.42	CALM4	5	6	16	17	1.6	0.6	0.3	0.5	0.6	0.4	**
9906.12	DBI	6	6	18	18	0.8	0.7	0.8	0.4	0.6	0.4	*
9922.10	DBI	6	6	18	18	0.4	0.4	0.6	0.2	0.2	0.2	****
11785.07	FKBP1A	4	3	10	8	0.5	0.3	0.4	U	ND	ND	*
12366.14	MIF	2	1	6	1	ND	ND	U	0.1	ND	U	**