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Differential Expression of Proteins Related with Penile Apoptosis in a Rat after Cavernous Nerve Resection

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= Abstract =

Purpose: The mechanism including changes of proteome within cavernosal tissue after cavernous nerve injury were not evaluated. We performed proteomics and functional analysis to identify proteins of penile corpus cavernosum whose expression was or was not altered by cavernous nerve resection (CNR).

Materials and Methods: Using 8-week-old male WKY rats, sham and CNR operation under microscope were performed. After 8 weeks, penile tissues of sham and CNR group were harvested. We used 2-DE and MALDI-TOF/TOF (AB 4700) to identify of differently expressed penile proteins. 2-DE gels were stained with silver nitrate and the gels were analyzed with PDQuest.

Results: We isolated more than 950 proteins on silver-stained gels of whole protein extracts from normal rat penile corpus cavernous. Of these proteins, 48 prominent proteins were identified using MALDI-TOF/TOF. Protein characterization revealed that the most prominent penile corpus cavernous proteins were those with antioxidant, chaperone, or cytoskeletal structure. Moreover, 11 proteins having levels elevated by CNR were annexin proteins, endoplasmic reticulum protein 29, glutathione s-transferase w-1, and others. In addition, Rho-GDP dissociation inhibitor (RhoGDI), a regulator of Rho proteins, was also increased in CNR rats compared with sham-operated control rats.

Conclusions: The apoptotic signals observed in penile tissues was greatly increased in CNR rats than in sham-operated rats. These results suggest that RhoGDI is one of the proteins regulated by CNR in penile smooth muscle strips, and has a crucial role in the early stage of penile apoptosis.

Key Words: Erectile dysfunction, Proteomics, RhoGDI

Introduction

Despite the decreased morbidity of radical prostatectomy (RP), erectile dysfunction (ED) remains one of the major RP-related complications and occurs in 15 to 40 percents of men under age 60 after surgery.[1-3] The wide disparity in outcomes has been explained by differences in surgical technique, underlying endothelial dysfunction, patient risk factors, and varying anatomy of cavernous nerve (CN) in humans.[4-6] Recent da-

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ta suggest that interindividual variation of cavernous nerve course account for the disparate potency rates after nerve-sparing RP.[7] Therefore the etiologies of ED after nerve-sparing RP seem to be related to diverse degree of unintentional injury to these variable nerve fibers.[8]

Therefore, CN injury is believed to be the main cause of ED following RP.[6] CN injury in rats has become the gold standard model for the assessment of ED after CN injury.[9] Recent study shows that all CN injury method (neurotomy, bulldog crush, hemostat nerve crush) caused similar results in terms of the magnitude and consistency of hemodynamic alterations in corpus cavernosum.[10]

The molecular mechanisms of ED after nerve-sparing RP have not been fully elucidated. Early apoptotic change and late fibrosis in corpus cavernosum has been postulated as the cause of ED after CN injury.[11-13] Many specific upstream/downstream molecular events initiating penile apoptosis or fibrosis have been investigated during certain time point after CN injury.[13] These molecular events included changes in activity of transforming growth factor, hypoxia-inducible factor, nitric oxide synthase, c-jun N-terminal kinase, and p38 mitogen-activated protein kinase (MAPK).[14] Recently, we also showed that tadalafil, a phosphodiesterase-5 inhibitor, prevented CN denervation-derived penile apoptosis via the AKT and extracellular-regulated protein kinase (ERK) 1/2 dependent pathways.[9] User et al[12] demonstrated that in rats underwent bilateral CN transection, decreases in penile weight and DNA content were observed as well as an increase in sub-tunical smooth muscle cell apoptosis. In a rat model of cavernous neurotomy, Klein et al[11] demonstrated that after bilateral cauterization of the CN, apoptotic cell death occurred in the erectile tissue of the penis as early as 2 days following denervation.

Early initiation of penile rehabilitation after RP has been advocated to preserve structure and function of the corpora cavernosa, and reported improved erectile function in an animal and human model.[1,15] These improvements are accompanied with the changes of certain proteins in the corpus cavernosum. However, the mechanism of the structural change remains to be elucidated. Proteomics is a relatively new branch of biology and a useful tool that studies proteins and their subcellular distribution, quantity, modification and interaction state, catalytic activity, and structure in biological system.[16,17] The potential of proteomic technology deserves investigation to elucidate the mechanism of the ED following RP. We, therefore, used a proteomic technology to discover the early changes in corpus cavernosum in rat model with CN resection (CNR). In this study, we analyzed the whole proteins promptly expressed in penile tissue and proteins having level altered during CNR, and determined the relationship of this protein with penile apoptosis.

Materials and Methods

1. Animals and cavernous nerve resection

A total of 15, eight-week-old young adult male Wister-Kyoto (WKY) rats were randomized into 2 groups, including sham-operated control and bilateral CNR groups. All rats were allowed free access to water and food and animal rooms were illuminated according to a 12 hr light/dark cycle and were maintained at a temperature of 21±2°C and a relative humidity of 55±8%. All experiments were performed in accordance with the institutional guidelines of Konkuk University, Korea. The CNR and sham operation was performed under anesthesia with ketamine/xylzine (80 mg/kg : 8 mg/kg, IM). Through a lower midline incision, the bladder and major pelvic ganglion were identified with a surgical microscopy. After dissecting the fascia and fat from the dorsolateral lobe of the prostate, the CNs could be readily identified at the lateral surface of the membranous urethra. If any CN ancillary branches identified, CNs including ancillary branches located in between prostate lower margin and symphysis pubis were totally removed to prevent any possible re-innervation. Two weeks after the sham-operation and CNR, penile tissues were harvested under anesthesia with the ketamine/xylzine mixture. The skin overlying the penis was incised and bilateral penile crura were exposed by removing part of the ischiocavernous muscle and fascia to expose the bulbous

urethra. Dissection of the urethra was performed away from the corpus cavernosum of the penis. After removal of urethra, the penile tissues were obtained excluding the glans and cartilaginous penis and removing part of overlying ischiocavernous muscle. Harvested tissues were washed free of blood with PBS several times, and tissue samples were snap-frozen in LN_2 and maintained at $-80^{o}C$ until processing for future studies or immersed in 4% paraformaldehyde for immunohistochemistry analysis

2. 2-Dimensional electrophoresis

Penile strips were homogenized in 2-DE lysis buffer containing 8 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), 2% CHAPS, and 1×complete protease inhibitor cocktail (Roche Applied science, Nonnenwald, Penzberg, Germany). Homogenates was centrifuged at 12,000×g for 10 min at $10^{o}C$ and the supernatant was harvested. Samples were diluted with rehydration buffer containing 8 M urea, 0.28% DTT, 0.5% CHAPS, 10% glycerol, 0.5% appropriate ampholyte, and 0.002% bromophenol blue. 2-DE analysis was performed as reported in our previous studies. The density of silver-stained spots from 4 different experiment sets was detected and counted by both automation and manual spot-detection, and statistically analyzed with PDQuest software (Version 7.1.1, Bio-Rad, Hercules, CA, USA).

3. Mass spectrometry of protein spots

Protein identification was performed as described in our previous report. The protein spots were excised from the stained gel and digested with trypsin. The peptide samples were desalted using ZipTip C_{18} microtips (Millipore, Bedford, MA, USA), mixed with CHCA matrix solution and loaded onto stainless steel sample target plates. Peptide mass spectra were acquired by MALDI-TOF/TOF mass spectrometer (AB4700, Applied Biosystems) in the positive ion reflector mode. Spectra were processed with the Global Protein Server Explorer 3.0 software (Applied Biosystems). A combined MS and MS/MS analysis was performed using internal MASCOT (Matrix Science, London, UK) program. The resulting data were screened against rat databases downloaded from NCBI and the Swiss-Prot/ TrEMBL homepages.

4. Tunel assay

Tissue samples isolated were immersed in 4% paraformaldehyde for 6 hours, embedded in paraffin, and sectioned. The terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed using the ApopTag (R) Peroxidase In Situ. Apoptosis Detection Kit (S7100, Chemicon) on paraffin block sections (N=15). The sections were incubated with 0.05% DAB for 1 minute and then counterstained with Meyer's hematoxylin. The TUNEL assay was considered positive if brown color was noted in the nucleus. The numbers of apoptotic cells in corpus cavernosum tissue were counted at a screen magnification of ×400 in six different regions per penis and we analyzed five sections per animal.

5. Western blotting

Tissues were lysed with an extraction buffer (50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 5 mM DTT, 300 μM phenylmethyl sulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM NaF, 2 mM Na_3VO_4, 5 μg/ml aprotinin, 5 μM leupeptin, 1% Triton X-100, 10% glycerol, and 150 mM NaCl). Lysates were centrifuged (16,000×g, 10 min, at $4^{o}C$), and the supernatants collected were diluted 1 : 1 (v/v) with SDS sample buffer containing 40 mM Tris-HCl (pH 6.8), 8 mM EGTA, 4% 2-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, and 4% SDS, and then boiled for 5 min. Proteins (20 μg/lane) were separated using 12% polyacrylamide SDS gels, and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore). The membrane was then blocked for 1 h and then incubated with anti-RhoGDI and -α-tubulin (Cell Signaling) antibodies diluted 1 : 2,000～5,000 overnight at $4^{o}C$. Immune complexes were detected with horseradish peroxidase-conjugated antibodies (Amersham-Pharmacia, Piscataway, NJ, USA) diluted 1 : 1,000 and incubated for 1 h at room temperature. After application of the secondary antibody, blots were incubated in enhanced chemiluminescence kits (Amersham-Pharmacia) and exposed to photographic film. Band

intensity was measured by computer analysis using Quantitation software (Bio-Rad, Hercules, CA, USA).

6. Data analysis

Data are presented as means±S.E.M. The statistical evaluation of data was performed using Student's t tests for comparisons between pairs of groups. A value of $p < 0.05$ was considered to be a statistically significant difference.

Results

1. CNR-induced penile corpus cavernosum apoptosis

Apoptotic cell death in penile corpus cavernosum tissue of CNR rats was confirmed with immunohistochemical analysis. Apoptotic cells were observed in penile tissues which was greatly increased in penile corpus cavernosum from rats after 2-week of CNR compared with sham-operated control (n=5, Fig. 1). This showed a similarity with the result previously reported by us.[9]

2. Whole protein expression in penile penile corpus cavernosum

Whole proteins isolated from penile tissue were visualized by silver-stained 2-DE gel in normal WKY rats. Fig. 2 shows a typical 2-DE gel of total proteins from rat penile corpus cavernosum tissues. More than 950 proteins may be seen in gel, with isoelectric pH

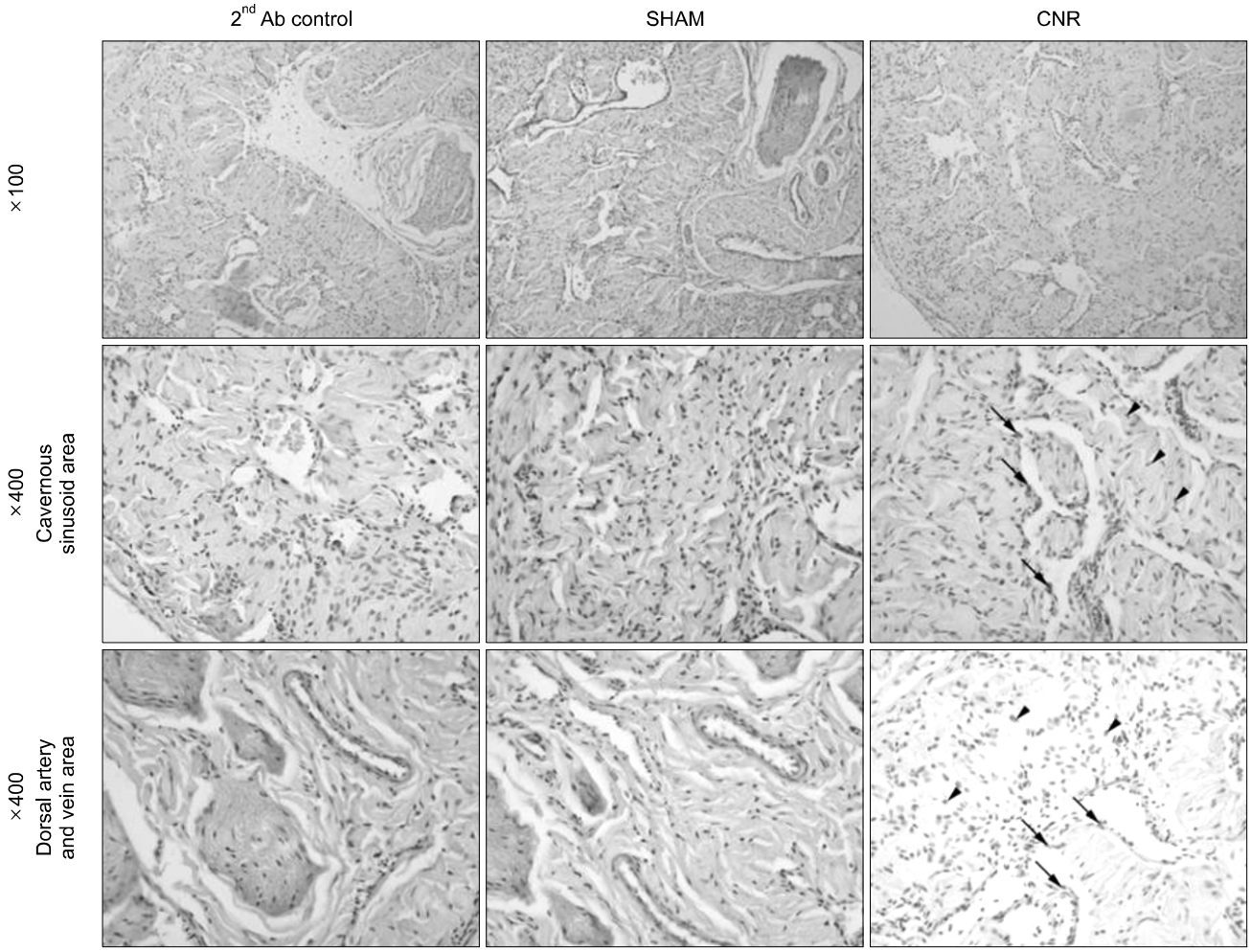

Fig. 1. Increment in apoptosis of penile corpus cavernosum from CNR rats. Immunohistochemical staining shows higher immunoreactivity in endothelial cells (arrows) and in smooth muscle cells (arrowheads) of CNR group than those of control group. The results are typical examples in 5 independent experiments. 2nd Ab: secondary antibody control. Magnification ×100 or ×400.

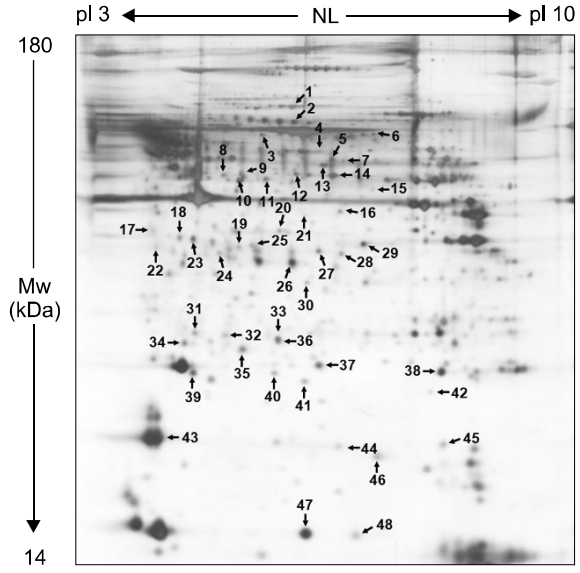

Fig. 2. Images of a silver-stained 2-DE showing whole proteins identified in rat penile corpus cavernosum strips. Proteins were subjected to IEF on IPG strips (pH 3～10, nonlinear, 17 cm) and then separated by 12% SDS-PAGE. The protein spots were visualized with silver stain. The arrows indicate proteins detected with MALDI-TOF/TOF. The numbers indicated in the gels correspond to the numbers in Tables 1. The results are typical examples in 4 independent experiments.

values of pH 3～10 and MW of 14～180 kDa. The MALDI-TOF/TOF analyzer identified 48 prominent proteins. Table 1 shows identifies the proteins, highlights representative peptide sequences and the sequence coverage, notes the theoretical and experimental pI and MW values, gives accession numbers from both the Swiss-Prot and NCBI databases, and describes known functions. Protein identifications revealed that prominent penile corpus cavernosum.

Proteins included those with cytoskeletal, antioxidant, or chaperon functions, and that proteins with roles in muscle contraction, metabolism, and signal transduction were also evident. Endoplasmic reticulum proteins were also identified.

3. Changed expression in penile proteins between CNR and sham rats

Fig. 3 show the expression of protein in whole proteomes from penile corpus cavernosum strips of CNR rats and sham rats harvested at 2 weeks after operation. The protein expression was analyzed using PDQuest. Expressions of 11 proteins showed to be increased in penile corpus cavernosum tissues from CNR

Table 1. Summary of proteins prominently expressed in penile tissue from WKY rats as identified by MALDI-TOF/TOF

No	Protein name	Peptide sequences	Score/ SC (%)	Protein pI/*Mr* (kDa) (Experimental)	Accession No /Database	Known function
1	Gelsolin	VSETRPSTMVVEHPEFLK EVQGFESSTFQGYFK HVVPNEVVVQR VSNSGGSMSVSLVADENPFAQSALR SEDCFILDHGR TPSAAYLWVGTGASDAEK AQHVQVEEGSEPDGFWEALGGK	476/15	5.76/86.0 (5.76/101.5)	51854227/NC P13020/SP	Actin-modulating protein
2	Hemopexin protein	GEFVWR LFQEEFPGIPYPPDAAVECHR GECQSEGVLFFQGNR FNPVTGEVPPR DYFISCPGR GATYAFSGSHYWR SGAQATWAELSWPHEK	457/15	7.58/51.3 (5.76/88.9)	60688311/NC P20059/SP	Heme binding protein
3	Dihydrolipoamide S-acetyltransferase	VPLPSLSPTMQAGTIAR ILVPEGTR VPEANSSWMDTVIR	142/7	5.70/58.7 (5.53/80.9)	11580/NC P08461/SP	Pyruvate dehydrogenase activity

Table 1. Continued

No	Protein name	Peptide sequences	Score/ SC (%)	Protein pI/*M*r (kDa) (Experimental)	Accession No /Database	Known function
4	Serum albumin precursor	FPNAEFAEITK LGEYGFQNAILVR APQVSTPTLVEAAR CCTLPEAQRR PCFSALTVDETYVPK AADKDNCFATEGPNLVAR	342/13	6.09/68.7 (5.83/74.2)	55391508/NC P02770/SP	Body fluid osmorregu-lation
5	Chaperonin containing TCP1, subunit 2 (beta)	KIHPQTIIAGWR IHPQTIIAGWR HGINCFINR GATQQILDEAER	162/6	6.01/57.4 (5.85/65.8)	53733839/NC P80314/SP	Molecular chaperone
6	WD repeat1 protein	VFASLPQVER LATGSDDNCAAFFEGPPFK MTVDESGQLVSCSMDDTVR	133/13	6.21/66.1 (6.21/83.5)	20269306/NC O88342/SP	Disassembly of actin filaments
7	Selenium binding protein 1	EEIVYLPCIYR SPHYSQVIHR IYVVDVGSEPR	100/6	6.10/52.5 (5.87/66.9)	18266692/NC	Selenium binding protein
8	Serine (or cysteine) proteinase in-hibitor, clade F, member 1	LAAAVSNFGYDLY SSFVAPLE TTLQDFHLDED YGLDSDLNC AAFEWNEEGAGTSSNPDLQPV DTDTGALLFIGR	376/18	6.04/46.4 (5.21/59.2)	29293811/N P97298/SP	Neuronal differ-entiation
9	Serine (or cysteine) proteinase inhibitor, clade F, member 1	LAAAVSNFGYDLY SSFVAPLE KTTLQDFHLDED TTLQDFHLDED YGLDSDLNC AAFEWNEEGAGTSSNPDLQPV DTDTGALLFIGR	534/19	6.04/46.4 (5.36/62.6)	29293811/N P97298/SP	Neuronal differ-entiatio
10	Guanine deaminase	TPQLALIF IVFLEESSQQE EWCFKPCEI FQSTDVAEEVYT THDLYIQSHISEN LATLGGSQALGLD FLYLGDDR	433/17	5.48/50.9 (5.33/57.8)	7533042/N Q9WTT6/SP	Guanine de-aminase activity
11	Serine (or cysteine) proteinase inhibitor, clade F, member 1	LAAAVSNFGYDLY SSFVAPLE KTTLQDFHLDED TTLQDFHLDED AAFEWNEEGAGTSSNPDLQPV DTDTGALLFIGR	394/16	6.04/46.4 (5.57/57.6)	29293811/N P97298/SP	Neuronal differ-entiatio
12	Alpha enolase	EIFDS IGAEVYHNL YITPDQLADLY SCNCLLL VNQIGSVTESLQAC YNQILR	347/13	6.16/47.1 (5.77/60.8)	P17182/SP	Glycolysis

Table 1. Continued

No	Protein name	Peptide sequences	Score/ SC (%)	Protein pI/*Mr* (kDa) (Experimental)	Accession No /Database	Known function
13	Aldehyde dehydrogenase, mitochondrial	AAFQLGSPW TIPIDGDFFSYT TFVQEDVYDEFVE VVGNPFDS GYFIQPTVFGDV TIEEVVG ELGEYGLQAYTEVK	446/16	5.83/54.3 (5.83/64.4)	3121992/N P81178/SP	Carbohydrate and alcohol metabolism
14	Alpha enolase	EIFDS AAVPSGASTGIYEALEL YITPDQLADLY VNQIGSVTESLQAC YNQILR	367/13	6.16/47.0 (5.88/60.5)	P04764/SP	Glycolysis
15	Tu translation elongation factor, mitochondrial	KYEEIDNAPEE YEEIDNAPEE GITINAAHVEYSTAAR	215/6	7.23/49.5 (6.20/53.9)	27370092/NC	Protein biosynthesis
16	Actin-capping protein gCap39	QAALQVADGFIS YSPNTQVEILPQGR	146/7	6.73/39.2 (5.87/48.0)	109521/N P24452/SP	Cytoskeleton organization
17	Tropomyosin 2	CKQLEEEQQALQ IQLVEEELD IQLVEEELDRAQE KLVILEGELE TIDDLEDEVYAQK	465/17	4.66/32.8 (4.41/41.2)	11875203/N P58774/SP	Smooth muscle contraction
18	Preprohaptoglobin	GSFPWQA MGYVSGWG SCAVAEYGVYVR	175/10	7.16/30.0 (4.82/39.5)	204657/N P06866/SP	Hemoglobin binding protein
19	Preprohaptoglobin	VMPICLPSKDYVAPG MGYVSGWG SCAVAEYGVYVR	151/13	7.16/30.0 (5.34/37.1)	204657/N Q60574/SP	Hemoglobin binding protein
20	Alpha-actin	VAPEEHPTLLTEAPLNP GYSFVTTAE SYELPDGQVITIGNE DLYANNVMSGGTTMYPGIAD QEYDEAGPSIVHR	263/23	5.45/37.8 (5.67/41.7)	49864/N P68134/SP	Cytoskeleton organization
21	Alpha 2-actin	AVFPSIVGRP VAPEEHPTLLTEAPLNP GYSFVTTAE QEYDEAGPSIVHR	177/13	5.23/42.0 (5.79/46.0)	6671507/N P62737/SP	Cytoskeleton organization
22	Tropomyosin 1	RIQLVEEELD IQLVEEELD IQLVEEELDRAQE AEFAE SIDDLEDELYAQK	332/16	4.69/32.7 (4.61/37.1)	P58771/SP	Muscle contraction
23	Preprohaptoglobin	GSFPWQA VMPICLPSKDYVAPG MGYVSGWG MGYVSGWG SCAVAEYGVYVR	263/16	7.16/30.0 (4.95/39.1)	204657/N P06866/SP	Hemoglobin binding protein

Table 1. Continued

No	Protein name	Peptide sequences	Score/ SC (%)	Protein pI/*Mr* (kDa) (Experimental)	Accession No /Database	Known function
24	Pyruvate dehydrogenase (lipoamide) beta	EAINQGMDEELERDE EGIECEVINL VTGADVPMPYAK	180/10	6.41/38.9 (5.17/36.3)	18152793/N Q9D051/SP	Acetyl-Co A biosynthesis
25	Capping protein (actin filament) muscle Z-line, alpha 2	LLLNNDNLL EATDPRPYEAENAIESW NFWNG IQVHYYEDGNVQLVSHK	164/17	5.57/32.9 (5.44/37.3)	38322760/N P47754/SP	Cytoskeleton organization
26	Similar to osteoglycin precursor	ESAYLYA DFADMPNL MEEIRLEGNPIALG HPNSFICL RLPTGSYF	265/16	5.85/34.1 (5.76/35.5)	27683465/N Q62000/SP	Bone formation
27	Malate dehydrogenase, cytosolic	ENFSCLT NVIIWGNHSSTQYPDVNHA GEFITTVQQR	175/11	6.16/36.5 (5.83/36.3)	319837/N P14152/SP	Dihydropyrimidinase activity
28	Glycerol 3-phosphate dehydrogenase	VCIVGSGNWGSAIA IVGSNASQLAHFDPR	50/10	6.32/37.4 (5.87/36.5)	2317252/N O35077/SP	Glycerol synthesis
29	Aldehyde reductase 1	VAIDMGYR HIDCAQVYQNEK HIDCAQVYQNEKEVGVALQEK RQDLFIVSK AIGVSNFNPLQIER YKPAVNQIECHPYLTQEK LIEYCHCK TTAQVLIR HKDYPFHAEV	567/30	6.26/35.8 (6.03/39.3)	6978491/N P07943/SP	Carbohydrate metabolism
30	3-Mercaptopyruvate sulfurtransferase	HIPGAAFFDID AFGHHSVSLLDGGF SPSEPAEFCAQLDPSFI THEDILENLDA FQGTQPEP AQPEHVISQGR	382/25	5.88/32.9 (5.79/33.3)	55824737/NC P97532/SP	Sulfate transport
31	Heat shock protein 27	LFDQAFGVP AVTQSAEITIPVTFEAR	107/13	6.12/22.9 (4.95/28.2)	204665/N P42930/SP	Protein folding
32	Heat shock protein 1	RVPFSLL VPFSLL DWYPAHS LFDQAFGVP AVTQSAEITIPVTFEAR	252/20	6.45/22.9 (5.22/28.0)	7305173/N P14602/SP	Protein folding
33	Heat shock protein 27	RVPFSLL VPFSLL SPSWEPFRDWYPAHS DWYPAHS LFDQAFGVP VSLDVNHFAPEELTV AVTQSAEITIPVTFEAR	444/32	6.12/22.9 (5.66/27.2)	204665/N P14602/SP	Protein folding

Table 1. Continued

No	Protein name	Peptide sequences	Score/ SC (%)	Protein pI/*Mr* (kDa) (Experimental)	Accession No /Database	Known function
34	Myosin light polypeptide 3	AAPAPAAAPAAAPEPERP EAFQLFD ITYGQCGDVL ALGQNPTQAEVL DTGTYEDFVEGL HVLATLGER	395/36	5.03/22.1 (4.87/26.9)	6981240/N P16409/SP	Regulatory light chain of myosin
35	Preproapolipoprotein A-I	DSGRDYVSQFESSTLG MQPHLDEFQE WNEEVEAY VVAEEF FGLYSDQM NHPTLIEYHTK	342/24	5.52/30.1 (5.37/26.3)	55747/N P04639/SP	Cholesterol transport
36	Peroxiredoxin 6	DFTPVCTTELG VVFIFGPDK LSILYPATTG NFDEILR	204/17	5.72/24.7 (5.66/27.1)	O08709/SP	Antioxdant activity
37	DJ-1 protein	GAEEMETVIPVDIM DVVICPDTSLEEAK	162/15	6.32/20.0 (5.82/25.6)	16924002/N O88767/SP	Redox-sensitive chaperon
38	Adenylate kinase 1	YGYTHLSTGDLL ATEPVISFYDKR	180/12	7.71/21.6 (7.67/24.8)	13242235/N P39069/SP	Cell growth
39	Peroxiredoxin 2	SLSQNYGVL NDEGIAY QITVNDLPVG SVDEALR	250/18	5.20/21.8 (4.94/24.8)	885932/N Q61171/SP	Antioxident activity
40	Adenine phosphor-ibosyltransferase	DISPLLKDPDSF IDYIAGLDSR	49/12	6.31/19.7 (5.63/24.9)	P08030/SP	AMP biosyn-thesis
41	Subunit d of mitochondrial H-ATP synthase	SWNETFHT LASLSEKPPAIDWAYY NCAQFVTGSQA KYPYWPHQPIENL	257/31	5.78/18.8 (5.79/24.3)	220904/N P31399/SP	ATP synthesis coupled proton transport
42	Alpha B-crystallin	RPFFPFHSPS APSWIDTGLSEM QDEHGFISR	193/18	6.84/19.9 (7.44/23.5)	57580/N P23928/SP	Protein folding
43	Fast skeletal muscle troponin C	SYLSEEMIAEF AAFDMFDADGGGDISV SEEELAECF NADGYIDAEELAEIFR	333/34	4.07/18.1 (4.87/20.7)	6678371/N P20801/SP	Muscle contraction
44	Cofilin, muscle iso-form	LLPLNDC YALYDATYETK	48/11	7.66/18.7 (5.65/20.0)	P45591/SP	Actin poly-merization
45	Adenylate kinase 1	YGYTHLSTGDLL ATEPVISFYDKR	111/12	7.71/21.6 (7.71/20.0)	13242235/N P39069/SP	Cell growth
46	Destrin	HFVGMLPEKDX HEYQANGPEDLNR	89/15	8.20/18.4 (6.36/19.3)	Q9R0P5/SP	Actin poly-merization

Table 1. Continued

No	Protein name	Peptide sequences	Score/ SC (%)	Protein pI/*M*r (kDa) (Experimental)	Accession No /Database	Known function
47	Chain D, rat transthyretin	TADGSWEPFASG TAESGELHGLTTDEKFTEGVY FTEGVY ALGISPFHEYAEVVFTANDSGHR	355/48	6.04/13.1 (5.80/15.4)	3212535/N P02767/SP	Thyroid hormone-binding protein
48	Similar to protein kinase C inhibitor	AQVAQPGGDTIFG IIFEDDR	70/17	5.91/13.4 (5.91/15.3)	34868506/N P70349/SP	Hydrolysis of adenosine 5'-monophosphoramidate substrates

The identity of each protein is shown. The names of the proteins, peptides representative of the identified sequences and the sequence coverage (SC), theoretical and experimental pI and *M*r values, accession numbers in both the SWISS-PROT (SP) and NCBI (NC) databases, and known functions are presented.

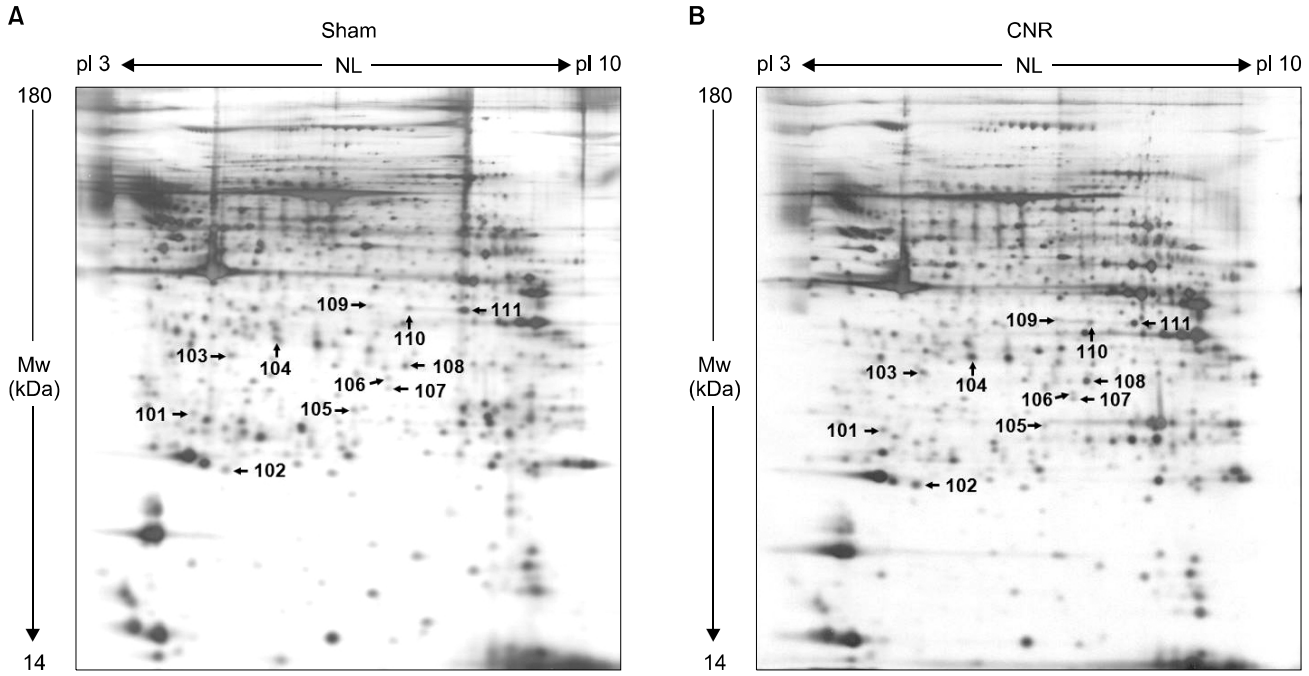

Fig. 3. Silver-stained 2-DE gels in whole proteins of penile corpus cavernosum strips from sham (A) and CNR (B) rats. Proteins were subjected to IEF on IPG pH 3～10 nonlinear (NL) strips and then separated by 12% (w/v) polyacrylamide SDS-PAGE. Arrows show proteins subsequently examined by MALDI-TOF/TOF. The numbers indicated in the gels correspond to the numbers in Table 2. The images are typical of those obtained in gels from 4 independent experiments.

rats compared with those from sham-operated control rats (n=4, Fig. 4 and 5A). Annexin A4 (spot 103), endoplasmic reticulum protein 29 (ERp29, spot 105), glutathione s-transferase ω-1 (GST ω1, spot 107), phosphatidylethanolamine binding protein (spot 102), and RhoGDI (spot 101) were up-regulated at all samples in CNR rats. Annexin A1 (spots 110 and 111) showed different isoforms with two pI values. These protein spots corresponding with annexin A1 were up-regulated in CNR rats compared with those from

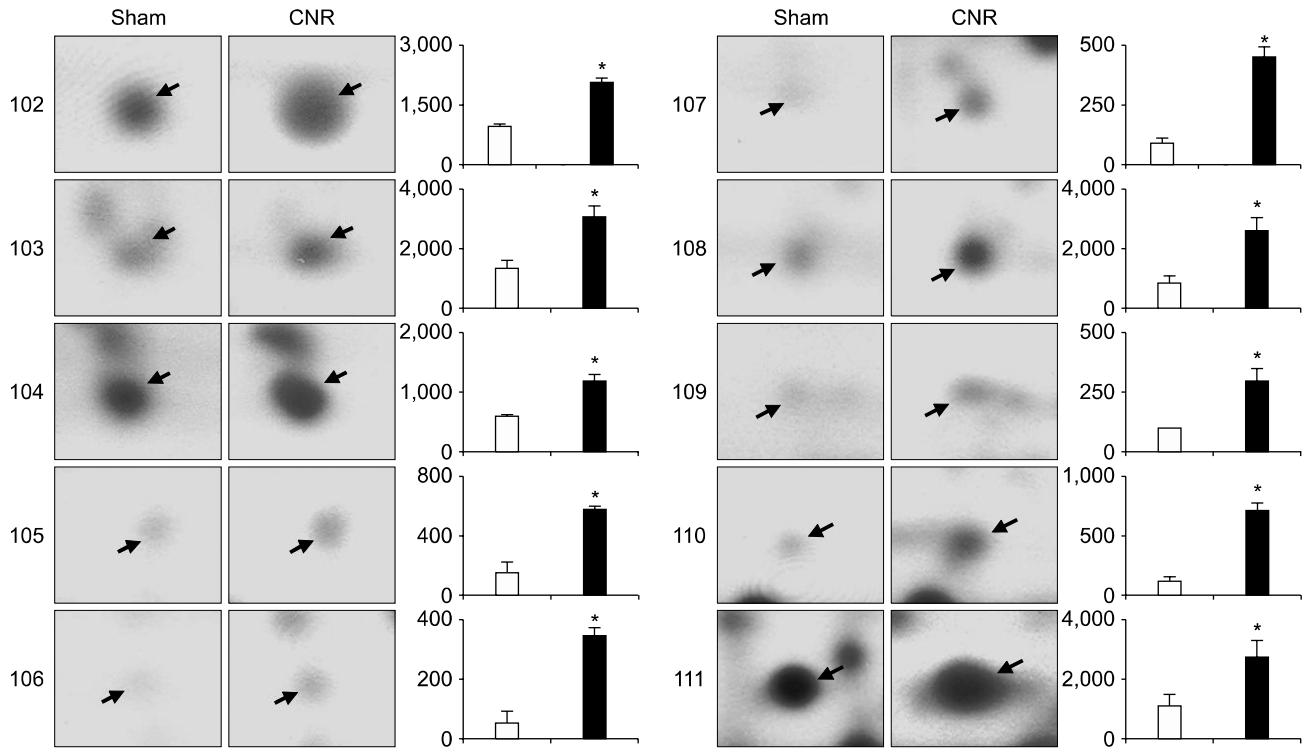

Fig. 4. Enlargement of a silver-stained 2-DE image showing rat penile corpus cavernosum proteins changed by CN resection. The arrowheads in both the left (sham control) and right (CNR) panels indicate proteins up-regulated compared with controls. The numbers indicated in the gels correspond to the numbers in Fig. 3 and Table 2. Regions containing proteins with statistically significant changed levels following the CNR were cropped and enlarged for this figure (*$p < 0.05$). Statistical analysis was performed on gels from 4 independent experiments using PDQuest. White bars, sham control; black bars, CNR.

sham-operated control rats. Table 2 shows the properties of the altered proteins identified with a MALDI-TOF/TOF analyzer, including fold changes, highlights representative peptide sequences and the sequence coverage, notes the theoretical and experimental pI and Mr values, and gives accession numbers from both the SWISS-PROT and NCBI databases.

4. Confirming RhoGDI expression in penile strips from CNR rats

It has been known that apoptosis of penis tissue is involved in penile disorders including ED and that Rho proteins, a small GTPase family, participate to the elevation of cell apoptosis.[9] Therefore, we selected RhoGDI, an important functional regulator of Rho proteins, to further evaluate interaction between expression and function in penis corpus cavernosum during CNR. First, to confirm the changes of RhoGDI in the CNR rats, we compared the expression in CNR and sham rats using a RhoGDI specific antibody. The results of expressions of RhoGDI in western blotting analysis showed to increase in penile corpus cavernosum strips from CNR rats compared with sham rats (n=5, Fig. 5B). The expression of α-tubulin was not changed in CNR rats.

Discussion

1. Characters of protein expression in penile tissue

In this study, we isolated more than 950 different proteins and partially mapped, 48 prominent proteins, the proteome from rat penile corpus cavernosum using 2-DE and MALDI-TOF/TOF. The rat penile proteins commonly found was consisted with antioxidant, cytoskeleton, chaperon, or contraction proteins. Especially,

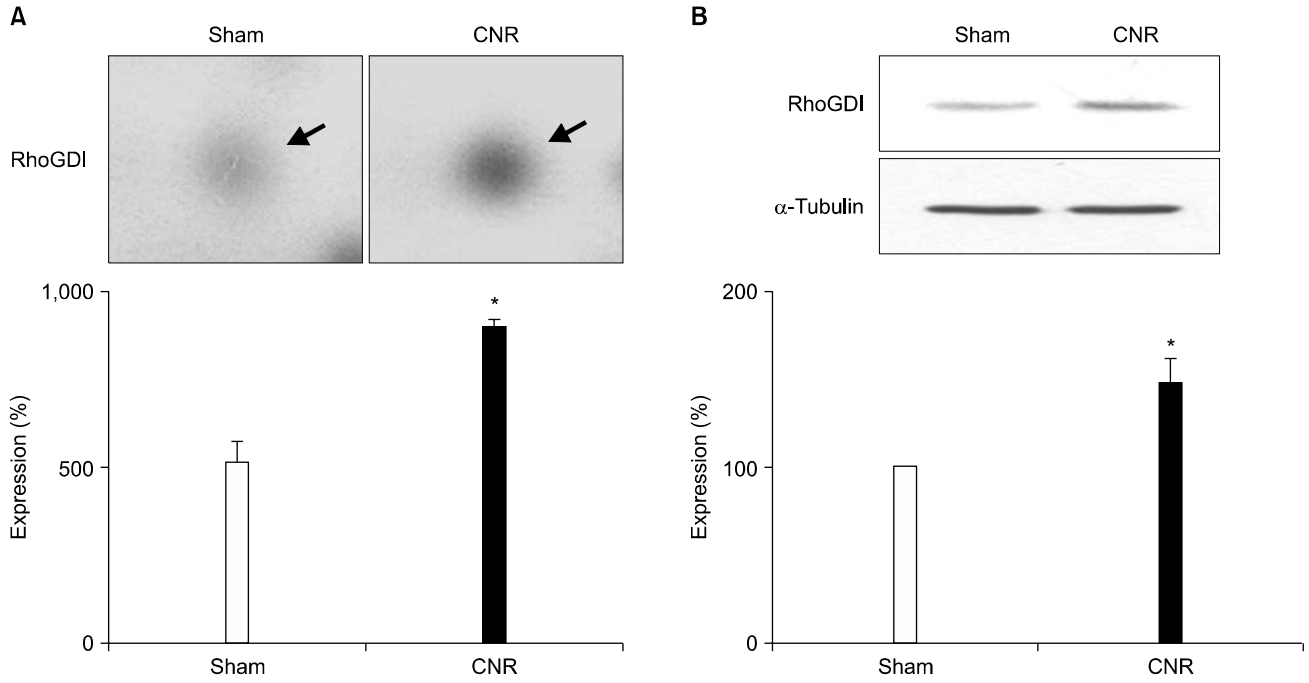

Fig. 5. Change of RhoGDI in penile corpus cavernosum strips from CNR rats. (A) Enlargement of a silver-stained 2-DE image showing RhoGDI changed by CNR as described in Fig. 4. (B) Western blot analysis using 1-DE gels. The separated proteins were immunoblotted with anti-RhoGDI or α-tubulin antibodies. Right panels; statistical analysis from left panels. The bands (n=5) of the RhoGDI in the sham control strips were defined as 100%. Denotes significant differences between the sham and CNR rats (*$p < 0.05$). The arrow indicates RhoGDI.

Table 2. Summary of proteins up-regulated in the penile tissue in CNR rats compared with sham-operated WKY rats. Proteins of penile tissue were quantified in CNR and sham-operated WKY rats

No	Δ	Protein name	Peptide sequence	Score/ SC (%)	Protein pI/*Mr* (kDa) (Experimental)	Accession No /Database	Known function
101	+1.7	Rho GDP-dissociation inhibitor 1	SIQEIQELDKDDESL QSFVLKEGVEY YIQHTY IDKTDYMVGSYGPR AEEYEFLTPMEEAPK	316/33	5.12/23.4 (4.87/28.8)	56541074/NQ99PT1/SP	Inhibition of Rho family GTPase
102	+2.1	Phosphatidylethanolamine binding protein	VDYGGVTVDELG VLTPTQVMNRPSSISWDGLDPG LYTLVLTDPDAPS FREWHHFLVVNM GNDISSGTVLSEYVGSGPP FKVESFR	326/41	5.48/20.7 (5.10/24.5)	8393910/NP31044/SP	Endoprptidase inhibitor activity
103	+2.0	Annexin A4	AASGFNATEDAQVL INQTYQQQYG SLEEDICSDTSFMFQ NKPAYFAER	217/17	5.31/35.9 (5.15/34.1)	37999910/NP55260/SP	Calcium/ phospholipid binding protein
104	+2.3	osteoglycin precursor	ESAYLYA DFADMPNL LSFLYLDHNDLESVPPNLPESL MEEIRLEGNPIALG HPNSFICL RLPTGSYF	272/24	5.85/34.0 (5.50/35.6)	27683465/NQ62000/SP	Bone formation

Table 2. Continued

No	Δ	Protein name	Peptide sequence	Score/ SC (%)	Protein pI/*M*r (kDa) (Experimental)	Accession No /Database	Known function
105	+3.8	Endoplasmic retuclum protein 29	FDTQYPYGEKQDEFK DGDFENPVPYSGAVK ILDQGEDFPASELAR	204/17	6.23/28.5 (5.84/29.1)	16758848/NP52555/SP	Intracellular protein transport
106	+6.5	Proteasome subunit alpha type 1	NQYDNDVTVWSPQGR FVFDRPLPVSR	63/9	6.15/29.5 (5.90/32.1)	38328483/NP18420/SP	Peptidase activity
107	+5.1	Glutathione S-transferase omega 1	GSAPPGPVPEGQIR FCPFAQR	71/8	6.54/27.7 (5.90/31.5)	50925894/NQ9Z339/SP	Glutathion metabolism
108	+3.0	Purine-nucleoside phosphorylase	TAEWL HRPQVAVICGSGLGGLTA SCVMMQG FHMYEGYSLS DHINLPGFCGQNPL FPAMSDAYD FPAMSDAYDRDMR	346/15	6.46/32.3 (6.07/33.2)	34869683/NC	Purine nucleoside phopshorylase activity
109	+3.0	NADH dehydrogenase 1 alpha subcomplex 10-like protein	LQSWLYASR IYDSFR	51/4	7.14/40.5 (5.86/42.5)	30171809/NCQ99LC3/SP	Mitochondrial electron transport
110	+5.5	Annexin A1	TPAQFDADELR	37/3	6.97/38.8 (6.17/41.7)	235879/NP07150/SP	Lipid metabolism
111	+2.4	Annexin A1	GGPGSAVSPYPSFNPSSDVAALHK TPAQFDADELR GTDVNVFNTILTTR KVFQNYR VFQNYR	329/16	6.97/38.8 (7.13/41.6)	235879/NP07150/SP	Lipid metabolism

Increases of at least 1.5-fold in 4experiments were considered to be significant ($p < 0.05$). The names of the proteins, peptides representative of the identified sequences and the sequence coverage (SC), theoreticaland experimental pI and *M*r values, accession numbers in both the SWISS-PROT (SP) and NCBI (NC) databases, and known functions are presented.

the actin-related proteins, peroxiredoxins, and heat shock proteins were prominent in penile corpus cavernosum tissue. Previously, it has been reported that the penile tissues contain proteins associated with functions including energy metabolism, oxidative stress, cytoskeleton and several proteins.[17] Micro array analysis showed a diversity of RNA expression in penile tissue.[18] Similar protein expression was observed in vascular smooth muscle.[19,20] These results imply that the protein composition in rat penile corpus cavernosum shows to have similar pattern of expression to other tissues[19] and that these proteins may participate in the physiological and pathological functions of penis.[21]

2. Changes of RhoGDI protein during CNR

Injury to the CN results in significant changes including atrophy and fibrosis of penile corpus cavernosum.[13] ED induced by CN injury is associated with a reduction in penile length and loss of cavernous smooth muscle cells.[12] This atrophy and fibrosis has been attributed to apoptosis whereby a cell will activate an intracellular pathway that leads to the activation of caspases, DNA cleavage, and eventually cell

death.[22] Similar histologic changes have been observed in the human penis after radical prostatectomy. Thus denervation of the penis results in significant changes in penile morphologies and functions. Moreover, there is cumulated evidence to accept that CNR in animals become the gold standard model for the assessment of human ED.[9] It has been reported that CNR induced various responses in penile corpus cavernosum including apoptosis.[18]

CNR model showed several changes in functional and molecular aspects,[18] the functional characteristics, however, were not fully defined. In this study, we found that RhoGDI content was increased in penile corpus cavernosum form CNR rats compared with sham-operated rats, which was confirmed with Western blot analysis. RhoGDI is one of most important regulator of the activity of Rho proteins. RhoGDI specifically inhibits the activity of GDP/GTP exchange of Rho proteins, hence that acts as an endogenous inhibitor of Rho proteins via keeping the protein in inactive state.[23] It has been known that the Rho pathway is implicated in a variety of cell functions.[24] Rho proteins participate to the cytoskeleton rearrangement regulated by extracellular stimuli and apoptosis in a variety of cells.[25-27] Several studies revealed that Rho proteins implicate to the morphological changes during apoptosis.[25,26] Moreover, we confirmed in this study that CNR induces morphological altering and increases apoptotic responses in penile corpus cavernosum.[9,28] It has been reported that increased activity in RhoA protein and ROCK is interacted with age-associated ED and suggested that Rho inhibitor suitable for therapy of ED.[29,30] As described above, the expression level of RhoGDI was elevated in penile corpus cavernosum from CNR rats compared with control rats, which may consequently inhibit the activity of Rho proteins. Previously, we showed that CNR increased the activity of Rho protein.[9] These results imply that CNR may lead to the activation of Rho protein and this may result in the elevation of penile cell apoptosis. Taken together, it can be assumed that the increment of RhoGDI may play as a secondary defense mechanism that exerts a protective effect against Rho-mediated penile apoptosis by CNR.

3. Properties of protein changes during CNR

Furthermore, we found that annexins A4 and A1, ERp29, GSTω1, and phosphatidylethanolamine binding protein were up-regulated by CNR. Some of these changed proteins, as well as RhoGDI, were identified as being related to cell apoptosis. It was shown that annexin protein plays a role in pathophysiology of ED in diabetic animals.[28] Exogenous annexin I facilitated ROS-induced apoptosis of cells.[31] Annexin I mediated apoptosis signals from extracellular space through activation of caspase-3.[32] Moreover, a number of GST regulated the activity of MAPK that involved inducing cell apoptosis in a variety of cells.[33] We showed that CNR-derived penile apoptosis was mediated by the ERK pathways.[9] It has been shown that ERp29 is directly associated with the resistance to oxidative stress and participates to the apoptosis of cells.[34] These results imply that these proteins altered by CNR are tightly involved in the induction of apoptosis of rat penile corpus cavernosum. Further analysis must clarify the possible mechanism of these proteins in penile corpus cavernosum apoptosis and ED.

Conclusions

We isolated more than 950 proteins on silver-stained gels of whole protein extracts from rat penile corpus cavernosum strips and identified 48 prominent proteins. Moreover, 11 proteins having levels altered by CNR were identified in this study. RhoGDI was significantly increased in penile corpus cavernosum from CNR rats compared with sham-operated rats. The apoptotic cells observed was greater in penile corpus cavernosum from CNR than in sham rats. These results suggest that Rho protein change has an important role during early stage of apoptosis in corpus cavernosum, and RhoGDI can be a potent therapeutic target for CN injury-related neurogenic ED.

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